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WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA, PA.

VITELLIN, CASEIN, LIVER AND MUSCLE TISSUE AS SOURCES OF PHOSPHORUS FOR THE RACHITIC RAT [1]

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(Received for publication November 30, 1942)

Recent investigations in this laboratory (Krieger, Bunkfeldt, Thompson and Steenbock, '41) have shown that for calcification of bone the phosphorus of either yeast nucleic acid or soybean phosphatides is approximately as available as inorganic phosphorus. Phosphorus from any of these sources was much more readily available than from phytic acid. The addition of vitamin D improved the availability of phytic acid phosphorous more than that of inorganic phosphorus, yet even with this addition it was less available than inorganic phosphorus. The present report contains data on the availability of phosphorus from ovo-vitellin, casein, pork liver, pork loin and ovo-phospholipid.

EXPERIMENTAL

The basal ration employed was the low-phosphorus, low-calcium ration previously used (Krieger and Steenbock, '40). This had a percentage composition of glucose [2] 51.67, steamed egg white 18.0, cooked starch 20.0, rice bran concentrate [3] 4.0, calcium and phosphorus-free salts 1.33, and cottonseed oil 5.0.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Cerelose.

[3] Vitab.

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The composition of the salt mixture was as follows: NaCl 105, KCl 120, $MgSO_4$ 90, $MnSO_4$ 0.20, $CuSO_4 \cdot 5\,H_2O$ 0.39, $K_2Al_2(SO_4)_4 \cdot 24\,H_2O$ 0.09, NaF 0.57, KI 0.05, and ferric citrate 16.15 parts, respectively. The basal ration contained 0.04% phosphorus and 0.02% calcium as determined by analysis. All additions to this basal diet were made at the expense of part of either the glucose or the egg white, or both. The phosphorus additions were based on the assumption that an approximately optimal intake of phosphorus was secured when 100 gm. of ration contained 0.257 gm. of phosphorus (Krieger, Bunkfeldt and Steenbock, '40). The Ca/P ratios were calculated from the total calcium and phosphorus contents of the ration. The calcium content of the ration was adjusted as desired by the addition of calcium carbonate. When inorganic phosphorus was added, it was in the form of an equimolecular mixture of the mono- and di-potassium phosphates. Ten micrograms of β-carotene in cottonseed oil were given by dropper to each rat daily to provide vitamin A.

Littermate rats weighing 50–60 gm. were distributed among the groups of a given series with six animals in a group. The paired feeding technique was used, with distilled water fed ad libitum. After being kept on the diet for 4 weeks the animals were killed with ether, the radii and ulnae removed for inspection of metaphyses, and the femora used for determination of ash, after extraction with alcohol.

The phosphorus of egg yolk protein versus inorganic phosphorus

Egg yolk protein was prepared as follows: The yolks of hens' eggs were separated carefully from the whites by mechanical means. They were then stirred thoroughly and poured slowly in a thin stream into boiling 95% alcohol. After draining free from excess solvent the coagulated protein was extracted in a Soxhlet extractor with alcohol until colorless, and then for 24 hours more with ether to remove vitamin D as well as other lipids. The product was dried and ground in

a ball mill. It contained 0.86% total phosphorus and 0.01% inorganic phosphorus.

The egg yolk protein was fed to furnish ½ and ¼ optimal phosphorus at a Ca/P ratio of 3/1, and ¼ optimal phosphorus at a Ca/P ratio of 6/1. This latter level of phosphorus was also fed with 45 U.S.P. units of vitamin D (calciferol) per 100 gm. of ration. The calcium and phosphorus contents of the rations were checked by analysis by the methods of Meloche, Clifcorn and Griem ('33) and Fiske and Subbarow ('25), respectively.

It is evident (table 1, series I) that the phosphorus of egg yolk protein was decidedly less available than inorganic phosphorus. It took more than twice as much egg yolk phosphorus as inorganic phosphorus to produce the same degree of calcification. Even ½ optimal egg-yolk phosphorus produced much less ash than ¼ optimal inorganic phosphorus. The condition of the wrist bones supported these conclusions.

As it was possible that the treatment employed in the isolation of the protein reduced the availability of its phosphorus, a second preparation was made without the use of high temperatures or alcohol. The yolks of 300 chicken eggs were dissolved in an equal volume of 10% saline solution and then extracted with ether to remove most of the lipids and pigments. The solution was then dialyzed in large cellulose acetate bags in the presence of toluene against cold tap water (6°C.) until no further precipitation resulted. The protein was filtered off, dried in a vacuum desiccator, then extracted continuously with ether at 25°C. for 2 days, and finally dried in vacuo at room temperature. The preparation contained 1.28% phosphorus. This was higher than the first product which contained 0.86% phosphorus, due to the fact that it had been freed from the water soluble protein livetin. Livetin has been reported to make up one-fourth of egg yolk protein and to contain only 0.05% phosphorus as contrasted with 1.30% phosphorus for vitellin (Kay and Marshall, '31).

TABLE 1

Availability of phosphorus from various sources[1]

SERIES AND SOURCE OF PHOSPHORUS	AMOUNT OF PHOSPHORUS AND Ca/P RATIO[2]		FOOD CONSUMED	GAIN IN WEIGHT	WEIGHT OF ALCOHOL-EXTRACTED FEMUR	FEMUR ASH	
	gm.	*Ca/P*	*gm.*	*gm.*	*gm.*	*gm.*	%
I.							
1. Egg yolk protein	0.128	3/1	188	25	0.0811	0.0225	27.7
2. Egg yolk protein	0.064	3/1	187	28	0.0832	0.0213	25.6
3. Egg yolk protein	0.064	6/1	186	28	0.0824	0.0205	24.9
4. Inorganic salts	0.064	3/1	190	34	0.0969	0.0351	36.2
5. Inorganic salts	0.064	6/1	189	31	0.0892	0.0287	31.2
6. Egg yolk protein[3]	0.064	6/1	188	27	0.0826	0.0316	38.3
II.							
7. Ovo-vitellin	0.128	3/1	197	56	0.1197	0.0414	34.6
8. Ovo-vitellin	0.064	3/1	197	49	0.1094	0.0347	31.7
9. Ovo-vitellin	0.064	6/1	197	47	0.0982	0.0278	28.3
10. Inorganic salts	0.064	3/1	198	51	0.1192	0.0476	39.9
11. Inorganic salts	0.064	6/1	195	43	0.1182	0.0403	34.2
12. Ovo-vitellin[3]	0.064	6/1	197	35	0.1251	0.0519	41.5
III.							
13. Casein	0.128	3/1	185	25	0.1256	0.0520	48.5
14. Casein	0.064	3/1	180	28	0.1096	0.0400	36.3
15. Casein	0.064	6/1	183	24	0.0989	0.0352	35.6
16. Inorganic salts	0.064	3/1	183	26	0.1004	0.0375	37.1
17. Inorganic salts	0.064	6/1	183	28	0.1038	0.0359	34.6
18. Casein[3]	0.064	6/1	182	18	0.1037	0.0450	43.4
IV.							
21. Pork liver	0.128	3/1	198	47	0.1190	0.0490	41.2
22. Pork liver	0.064	3/1	199	48	0.1056	0.0325	30.8
23. Pork liver	0.064	6/1	199	46	0.1098	0.0315	28.7
24. Inorganic salts	0.064	3/1	195	52	0.1107	0.0360	32.6
25. Pork liver[3]	0.064	6/1	198	48	0.1281	0.0537	41.9
V.							
26. Pork loin	0.128	3/1	170	39	0.1194	0.0479	40.1
27. Pork loin	0.064	3/1	170	42	0.1116	0.0342	30.6
28. Pork loin	0.064	6/1	170	40	0.1024	0.0297	29.0
29. Inorganic salts	0.064	3/1	170	42	0.1144	0.0385	33.6
30. Pork loin[3]	0.064	6/1	170	37	0.1194	0.0482	40.3
VI.							
31. Ovo-lipids	0.128	3/1	174.5	51.1	0.1219	0.0510	41.8
32. Ovo-lipids	0.064	3/1	173.7	44.8	0.0991	0.0324	32.7
33. Ovo-lipids	0.064	6/1	173.3	47.8	0.1056	0.0313	29.6
34. Inorganic salts	0.064	3/1	173.0	46.5	0.1118	0.0381	34.1
35. Ovo-lipids[3]	0.064	6/1	172.5	44.3	0.1271	0.0530	41.6

[1] Values are for an average of a group of six rats. The animals weighed 50–60 gm. initially and remained on the diets 28 days. It should be noted that any existing differences in the availability of the phosphorus in the sources tested from that of inorganic phosphorus were accentuated by feeding high calcium diets.

[2] Egg yolk protein, vitellin, and casein substitutions were for an equal weight of egg white. The pork preparations were substituted at the expense of the egg white and glucose so as to maintain a level of 18.0% protein in the diet. Substitutions indicated are per 100 gm. of the basal ration. The small amounts of calcium and phosphorus in the basal ingredients were included in the calculation of the calcium/phosphorus ratio.

[3] Plus 45 U.S.P. units of D_3 (calciferol) per 100 gm.

The second series of experiments duplicated the first, except for a reduction in the amount of protein used to provide the same amount of phosphorus. The results for equivalent amounts of phosphorus (table 1, series II) were the same as in the first series.

Casein phosphorus versus inorganic phosphorus

Casein was prepared without the use of alkali in the following manner: Dilute hydrochloric acid was added with stirring to fresh skimmed cows' milk diluted with an equal volume of water to complete precipitation. The precipitate was allowed to settle, then it was siphoned free from excess of supernatant liquors and washed seven times with water in a cold room at 6°C. The last three washings were done with distilled water. The preparation was then extracted with alcohol in a large Soxhlet extractor for 2 days followed by extraction with ether for 2 days. The product when dried contained 0.74% total phosphorus and 0.01% inorganic phosphorus.

The experimental feeding technique was identical with that of the ovo-vitellin series. The results (table 1, series III) show that the availability of casein phosphorus was about the same as that of inorganic phosphorus.

Pork liver phosphorus versus inorganic phosphorus

Pork liver freed from connective tissue was dried at 70°C. for 24 hours under vacuum, then extracted continuously with ether-alcohol for 48 hours and with ether for 24 hours. It contained 82.0% protein, 1.14% total phosphorus, 0.59% inorganic phosphorus and practically no lipids. Analyses show that less than 13.0% of the phosphorus of the dried liver had been removed by the extractions.

The availability of this pork liver preparation was tested in an experimental series similar to the vitellin series except that it included only one inorganic control. The results (table 1, series IV) indicate that the phosphorus of this liver prep-

aration was slightly less available than inorganic phosphorus. The addition of vitamin D was very beneficial.

Pork loin phosphorus versus inorganic phosphorus

The method of preparation of the pork loin sample was identical with that used for the liver preparation. The dried sample after ether-alcohol extraction contained 85.0% protein, 0.75% total phosphorus, 0.50% inorganic phosphorus and practically no lipids. The availability of the phosphorus of this pork loin preparation was tested by an experimental series similar to that of the liver series. The results (table 1, series V) show that the phosphorus of the pork loin preparation was definitely less available than inorganic phosphorus.

Ovo-phospholipid phosphorus versus inorganic phosphorus

The phospholipid preparation was obtained by ether and alcohol extraction of the yolks from hens' eggs. The solvents were removed under reduced pressure, and the residue taken up in ether and filtered. To the clear filtrate enough acetone was added to precipitate the phospholipids; these were stirred violently with acetone to yield a white wax-like product which was preserved under acetone, in a dark bottle in the cold. Freed from acetone, it contained 3.2% phosphorus and 4.5% nitrogen. To incorporate it in the ration it was taken up in ether solution which was evaporated on the glucose.

The results (table 1, series VI), show that the phosphorus of the lipids was slightly less available than inorganic phosphorus; one-fourth optimal phosphorus produced slightly less calcification and also a lighter bone than its inorganic control.

DISCUSSION

The literature on phospho proteins offers no explanation for the observed differences in the availability of the phosphorus of casein and vitellin, although a number of investigators have studied the effect of acids, alkalies and various enzymes on the organic phosphorus complexes contained in these proteins.

Rimington and Kay ('26) found that phosphoric acid was not cleaved from casein by either pepsin or 1% H_2SO_4 at 37°C. in 48 hours. However, it was liberated by pancreatic juice and 1% NaOH at 37°C. in 24 hours. Bone and kidney phosphatases produced only partial hydrolysis. Sodamitsu ('37) found that trypsin, free from phosphatases, did not liberate any phosphoric acid from casein. Rimington ('27) produced a phospho peptone by partial hydrolysis with trypsin. Levene and Hill ('33) believed that this phospho peptone consisted of serine and glutamic acid with the phosphoric acid in ester linkage with the serine. Rapoport ('37) determined that at least ⅓ of the phosphorus was not attached to serine. Sodamitsu ('37) concluded that phosphoric acid was combined in the casein molecule in various ways. Damodaran and Ramachandran ('40) by successive digestion of casein with pepsin and trypsin prepared an enzyme-resistant phospho-peptone which contained glumatic acid, isoleucine and serine.

The data on the phosphoric acid complexes of ovo-vitellin also are rather limited. Posternak ('28) believed that serine phosphoric acid constituted a phosphorus containing nucleus in ovo-vitellin. Lipmann and Levene ('32) prepared serine phosphoric acid from vitellinic acid which, in turn, was prepared from vitellin by digestion with 12% ammonia. Blackwood and Wishart ('34) from studies with pepsin and trypsin concluded that vitellin contained at least two widely dissimilar phosphoric acid complexes, of which one was very resistant to enzymic attack.

In view of the fact that Krieger, Bunkfeldt and Steenbock ('40) found the phosphorus of phytic acid to be available only with difficulty under experimental conditions similar to those used by the authors with ovo-vitellin, the possibility of the presence of phytic acid in this protein was entertained, especially since Rapoport ('40) has reported the presence of phytic acid in the tissues of the chicken, specifically in the erythrocytes. However, boiling with 1% KOH was found to liberate 50% of the phosphoric acid in 30 minutes and 100%

in 60 minutes from both casein and vitellin. Phytic acid is well-known to be stable under these conditions. Our preparation was entirely stable when boiled in 10% KOH for 1 hour. Furthermore, no phytic acid could be precipitated from a 10% KOH hydrolysis of vitellin by the technique of McCance and Widdowson ('35).

The lesser availability of the phosphorus of pork loin also is without explanation. Regarding pork liver and ovolipid phosphorus, the observed differences were not sufficiently pronounced to be considered factual. They probably should be regarded in the same light as the data previously obtained with soybean phosphatides (Krieger, Bunkfeldt and Steenbock, '40) which were assumed to show equality of value with inorganic phosphorus.

SUMMARY

Studies on the availability of phosphorus for calcification of bone in the rachitic rat on a high-calcium diet revealed that the phosphorus of ovo-vitellin is present in a different form from that occurring in casein. While it took more than twice as much vitellin phosphorus as inorganic phosphorus at a Ca/P ratio of 3/1 to produce the same degree of calcification, casein phosphorus was found equal in value to inorganic phosphorus under the same experimental conditions. The phosphorus from pork loin was definitely less available than inorganic phosphorus but not to the same degree as vitellin phosphorus. Pork liver and ovo-phospholipid phosphorus approximated inorganic phosphorus in value.

LITERATURE CITED

Blackwood, J. H., and G. M. Wishart 1934 The hydrolysis of lecitho-vitellin by pepsin and by trypsin-kinase. Biochem. J., vol. 28, pp. 550–558.

Damodaran, M., and B. V. Ramachandran 1940 Amino acids of casein phosphopeptone. Nature, vol. 145, p. 857.

Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, pp. 375–400.

Kay and Marshall cited by J. Needham 1931 The Macmillan Company, New York, Chemical Embryology, vol. 1, p. 294.

KRIEGER, C. H., R. BUNKFELDT, C. R. THOMPSON AND H. STEENBOCK 1941 Cereals and rickets. XIII. Phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification. J. Nutrition, vol. 21, pp. 213–220.

KRIEGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. The availability of phytic acid phosphorus. J. Nutrition, vol. 20, pp. 7–14.

KRIEGER, C. H., AND H. STEENBOCK 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutrition, vol. 20, pp. 125–132.

LEVENE, P. A., AND D. W. HILL 1933 On a dipeptide phosphoric acid isolated from casein. J. Biol. Chem., vol. 101, pp. 711–718.

LIPMANN, F., AND P. A. LEVENE 1932 Serinephosphoric acid obtained on hydrolysis of vitellinic acid. J. Biol. Chem., vol. 98, pp. 109–114.

MCCANCE, R. A., AND E. M. WIDDOWSON 1935 Phytin in human nutrition. Biochem. J., vol. 29, pp. 2694–2699.

MELOCHE, V. W., L. E. CLIFCORN AND W. B. GRIEM 1933 Determination of calcium in mineral mixtures. J. Assn. Off. Agri. Chem., vol. 16, p. 240.

POSTERNAK, S. 1928 Sur la limite de degradation des lectocyrines par la trypsine. Compt. rend. acad., vol. 186, pp. 1762–1765.

RAPOPORT, S. 1937 Zur Frage der phosphorbindung in phosphorprotein. Biochem. Z., vol. 289, pp. 420–424.

———— 1940 Phytic acid in avian erythrocytes. J. Biol. Chem., vol. 135, pp. 403–406.

RIMINGTON, C. 1927 The phosphorus of caseinogen. Constitution of phosphopeptone. Biochem. J., vol. 21, pp. 1187–1193.

RIMINGTON, C., AND H. D. KAY 1926 Some phosphorus compounds of milk. II. The liberation of phosphorus from caseinogen by enzymes and other agents. Biochem. J., vol. 20, pp. 777–790.

SODAMITSU, R. 1937 Uber die dephosphorierung des caseins durch die phosphatase. J. Biochem., (Jap.), vol. 26, pp. 341–358.

NUTRITIONAL DERMATOSES IN THE RAT

VIII. VITAMIN A DEFICIENCY [1]

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ONE TEXT FIGURE AND TWO PLATES (ELEVEN FIGURES)

(Received for publication October 8, 1942)

Hitherto descriptions of the vitamin A deficiency syndrome in the rat were based on observations of animals that had been conditioned by diets which were deficient in other essential aliments as well as in vitamin A. The principal complicating deficiencies in various experimental rations resulted from inadequate amounts of natural sources of the vitamin B complex, vitamin E, essential unsaturated fatty acids and fat. The purposes of this communication are: (1) To report the gross and microscopic observations of the skin of rats reared on a diet deficient only in vitamin A, insofar as it now is possible to concoct such a ration. (2) To investigate the influence of inadequacies in the vitamin B complex, fat and fatty acids on the signs of vitamin A deficiency.

EXPERIMENT I

THE "UNCOMPLICATED" SIGNS OF VITAMIN A DEFICIENCY PRODUCED BY A DIET CONTAINING 10% YEAST AS THE SOURCE OF THE VITAMIN B COMPLEX

To six groups of 21-day-old rats, totalling thirty-six animals and ranging in body weight from 25 to 40 gm., the following diet was fed: Vitamin-free casein,[2] 18 gm.; sucrose, 56 gm.;

[1] Aided by a grant from the Rockefeller Foundation Fluid Research Fund.

[2] S. M. A. Corporation.

McCollum salts (no. 51), 6 gm.; yeast [3] (assayed for its content of individual factors of the vitamin B complex), 10 gm.; pure lard,[4] 10 gm. One gram of choline chloride was incorporated into each kilogram of the ration. Each animal twice weekly received supplements of 2.5 mg. of alpha-tocopherol [5] to supply vitamin E and viosterol [6] equivalent to 460 units of vitamin D. To control groups of similar rats, the above diet and a supplement of alpha-tocopherol were fed and in addition each rat twice weekly received oleum percomorpheum [6] equivalent to 1400 units of vitamin A and 200 units of vitamin D. The experimental ration was calculated to assure the presence of all of the known essential aliments save vitamin A.

For the first 5 weeks after the diet was started the rate of growth of the deficient animals equalled that of the control group. The rats were lively and had good appetites. During the next one to three weeks, increase in weight was less well sustained (fig. 1 c) than that of the control group (fig. 1 b). During the sixth to tenth weeks rapid growth ceased. Some of the weight curves showed slight but continuous rises for the next few weeks (fig. 1c). During this period the first visible manifestations of the deficiency were observed; these were nasal signs. The rims of the nares were swollen and erythematous and the nares contained collections of serosanguinous exudate. Small irregularly shaped blood stains appeared on the tails as the result of contact with the bloody snouts. This sign is of value because it was often the first objective evidence of the deficiency. In some cases the involvement of the nares was not discernible until several days after the blood stains were observed on the tails. The first ocular signs were lacrimation and photophobia. In the inner canthi there were col-

[3] Anheuser-Busch, Inc. 2074 blend A, strain G dried brewers' type yeast. Assay report from Dr. James D. Veron of the Anheuser-Busch, Inc. Laboratory: Thiamine 61.0 and 62.7 International Units per gram, riboflavin 77.9 μg. per gram, pyridoxine 95.0 μg. per gram, nicotinic acid 0.307 mg. per gram, and pantothenic acid 153.3 μg. per gram.

[4] Esskay brand.

[5] Merck & Company.

[6] Mead Johnson and Company.

lections of small bloody crusts. A few days later the eyelids were swollen and there was a loss of hair on the lids (fig. 3). In no case was there wide extension of the alopecia to the circumocular or so-called "spectacle region." Frequently the eyelashes were matted together into tufts and there were collections of exudate on the margins, causing them to adhere. When the eyelids were forced apart and the collections of exudate gently wiped away, tufts of eyelashes were easily pulled out. During the next few weeks there were corneal

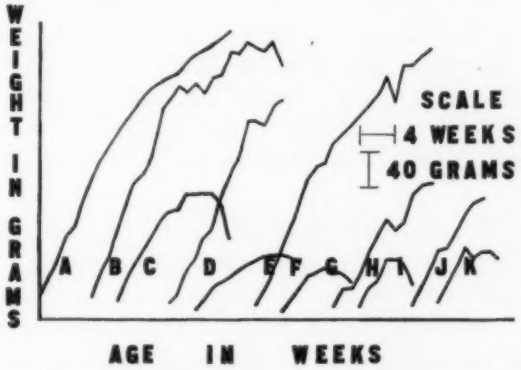

Fig. 1 Showing typical growth curves in experiments I, II, III and IV. (a) Normal McCollum male stock rat. (b) Vitamin A control rat; diet contained 10% yeast, 10% lard as the source of fat and indispensable unsaturated fatty acids, supplemental choline and vitamin E. (c) Vitamin A deficient rat; diet contained 10% yeast, 10% lard as the source of fat and indispensable unsaturated fatty acids, supplemental choline and vitamin E. (d) Vitamin A control rat; diet contained 5% yeast, 10% lard as the source of fat and indispensable unsaturated fatty acids, supplemental choline and vitamin E. (e) Vitamin A deficient rat; diet contained 5% yeast, 10% lard as the source of fat and indispensable unsaturated fatty acids, supplemental choline and vitamin E. (f) Vitamin A control rat; diet contained 5% yeast and 10% hydrogenated cottonseed oil as the source of fat and indispensable unsaturated fatty acids, no choline and no added vitamin E. (g) Vitamin A deficient rat; diet contained 5% yeast, 10% hydrogenated cottonseed oil as the source of fat and indispensable unsaturated fatty acids, no supplemental choline, and no added vitamin E. (h) Vitamin A control rat; diet contained no added source of fat or indispensable unsaturated fatty acids. (i) Vitamin A deficient rat; diet contained no added source of fat or indispensable unsaturated fatty acids. (j) Vitamin A control rat. United States Pharmacopoeia control diet. (k) Vitamin A deficient rat; United States Pharmacopoeia vitamin A assay diet.

opacities and more extensive ocular changes. The sides of the snout were edematous and erythematous and later were covered with serous honey-colored crusts. A few days after the onset of these signs the hair lacked luster and the coats were no longer sleek. The animals were less lively. They were tremulous and nervous; some were ataxic. Daily inspection of the skin showed no erythema, scaling or alopecia. As the disease progressed, the furs were slightly dishevelled (fig. 2), but there were no cutaneous signs indicative or diagnostic of vitamin B deficiency (Sullivan and Nicholls, '40a, b, '41, '42); pediculosis was not observed. With electric clippers the hair was shaved from the backs of the rats during various stages of the experiment. There was satisfactory and rapid regrowth of the shaved hair except during the week or two before death when all growth ceased and the weights were declining.

As the disease progressed further the rats were weak and slightly humped. Subcutaneous abscesses occurred in a few animals and abscesses of the tongue and buccal cavity were common. "Snuffles" characterized all of the groups and rales frequently were heard. With two exceptions, death occurred in from 10 to 15 weeks in the untreated animals. For 2 to 3 weeks prior to death there was a sharp decline in the weight curves (fig. 1c). Daily food consumption during the entire experimental period averaged 7 to 8 gm. per rat until a few days before death. During the last few days of life, the food consumption varied from 0 to 2 or 3 gm. The presence of keratinized material and abscesses at the base of the tongue (fig. 7) and in the buccal cavity probably accounted in the majority of instances for the decreased food intake when the rats were moribund. Administration of vitamin A resulted in an alleviation of the obvious symptoms and signs, but all of the treated animals were not restored to a completely normal state of health. Irving and Richards ('38) have described the severity and extent of pathologic alterations due to vitamin A deficiency. This should be emphasized because in

contrast to the action of the vitamin B complex in rats depleted of vitamin B, curative doses of vitamin A administered for 3 months to animals depleted of vitamin A were less efficacious. The "cured animals" were weak and subject to "cage mishaps" such as traumatizing the feet and the nose. The ataxia never entirely disappeared although it was less noticeable. The luster of the fur failed to reappear. By contrast, after the administration of vitamin B complex to groups of severely depleted vitamin B-deficient rats that appeared to be in extremis and incapable of surviving for another day, the response was striking. There was a complete restitution to an apparently normal state of health in 2 or 3 weeks after receiving vitamin B, and within a month it was not possible to distinguish by gross and/or microscopic means the formerly depleted animals from animals that had been normal since birth.

In an apparently resistant rat, the onset of signs of deficiency was delayed for 22 weeks and death occurred in the twenty-seventh week of the experiment. Pediculosis was observed in the twenty-sixth week of the experiment.

Microscopic examination

Numerous sections from every portion of the skin were examined. Sections also were made of the eyes, the paraocular glands, submaxillary glands, the tongue and the viscera. The examination of the epithelial tissues other than the skin established microscopic evidence of vitamin A deficiency. In rats showing evidence of vitamin A deficiency in the submaxillary glands, the eye, the tongue and/or the viscera, the following cutaneous changes were observed: There was hyperkeratosis; the lamellae were thin, wavy, loose and contained no intermingled crusts, or extruded keratohyalin granules. The epidermis in the early stages of the deficiency was of approximately normal thickness. In many sections there was dilatation of the hair follicles, particularly in the upper half of the shaft (fig. 4), where the lining of the follicle was

atrophic. Frequently the sebaceous glands attached to the upper part of the sides of the shaft were dilated and their cells were completely disintegrated. The lower portion of the follicle including the bulb remained intact and in many sections the hair was present and apparently structurally normal despite the dilatation and atrophy of the upper portion of the follicle, as a result of which there were dilated thin-walled biconcave spaces containing loosely arranged keratotic lamellae. In some instances (fig. 5) the hyperkeratotic material entirely filled the dilated spaces and in other cases it was insinuated between the wall of the follicle and a hair shaft that still remained (fig. 6). In some locations where the follicular dilation was superficial, wide and shallow lamellae were arranged in whorls under the layers which were disposed in parallel configuration. In many sections from the plantae there was a peculiar disturbance of the epidermal cells of the basal layer. Some of the basal cells showed disintegration and liquefaction and the basal layer was no longer clearly defined. In a few sections there were small patchy areas of acanthosis which alternated with the atrophic areas. Occasionally there were foci of epidermal cellular proliferation in the lower portion of the follicle below the atrophic region. In a few sections of the plantae there was extensive irregular acanthosis. There were no changes in the connective tissue. Diffuse mononuclear cellular infiltration in the cutis occurred in a few cases. It was most common on the sides of the snout; abscesses were rare. In the late stage there were dilatation, disintegration and atrophy of a few rudimentary coil glands, but the majority were intact; in numerous serial sections examined there was no evidence of a replacement of the columnar epithelium by squamous epithelium. As the disease progressed further there was dilatation, disintegration and atrophy of some of the sebaceous glands with subsequent wide dilatation in the bulbar region, and in some cases the epidermis was atrophic.

In the early stages the cutis was of approximately normal thickness. In some rats that had survived for long periods,

the cutis was thinner than that of the controls. There was a loss of subcutaneous fat. A constant abnormal finding was dilatation of the Meibomian glands' ducts. On the margins of the eyelids there were crusting and patchy areas of acanthosis. In the mucous membrane of the eyelids and lips, metaplastic epidermal changes were found. In the majority of the animals there were at the base of the tongue large, round and oval epithelial lined spaces filled with keratinized material. In many of the sections abscesses occurred in and around the keratinized tissue (fig. 7).

EXPERIMENT II

VITAMIN A DEFICIENCY COMPLICATED BY INADEQUACIES OF THE VITAMIN B COMPLEX

The diet used in experiment I contained a supplement of choline and 10% yeast, and was calculated to assure the presence of all of the well-known vitamins including E, and to provide all of the essential aliments. Many diets used in the past for the production of vitamin A deficiency contained 5% or less yeast as the source of vitamin B and lacked some other refinements attained in the diet described in experiment I. A few investigators incorporated yeast at higher levels than 5%, but the diets were deficient in other respects, namely, they contained insufficient or no fat, no vitamin E and no supplement of choline.

Gross and microscopic observations were made of the cutaneous alteration produced in rats by: (1) a vitamin A-deficient diet differing from that used in experiment I in two respects, namely, the yeast level was reduced from 10% to 5%, and the amount of sucrose was increased from 56 to 61 gm.; (2) a vitamin A-deficient diet containing 5% yeast and hydrogenated cottonseed oil as the source of fat.

A. Signs and symptoms produced by a diet containing 5% yeast as the source of the vitamin B complex and lard as the source of fat

To seventeen groups of 21-day-old rats totalling 102 animals and ranging in body weight from 25 to 40 gm., the

following diet was fed: Vitamin-free casein, 18%; sucrose, 60%; McCollum salts (no. 51), 6%; pure lard, 10%; yeast, 5%; choline, 1 gm. per kilogram of diet; viosterol equivalent to 460 units of vitamin D, twice weekly. For the first 2 weeks after the diet was started the rate of growth (fig. 1e) nearly equalled the rate of growth of the control group (fig. 1 d). The rats were lively and had good appetites. Thereafter there was an irregular and much less well-sustained increase in weight (fig. 1e). When the weight curves (fig. 1c) of the deficient rats in experiment I were compared with the weight curves (fig. 1 e) of the deficient rats in experiment II, it was apparent that the vitamin A-deficient rats that were reared on 10% yeast attained approximately 100% greater weight than those that were reared on 5% yeast. The relation of death to the weight curves was the same as that described in experiment I. The quantity of the food consumed was slightly less than that of the rats that were maintained on 10% yeast. The daily average intake during the experimental period varied from 5 to 7 gm. From the seventh to the ninth weeks there were cutaneous alterations indicative of vitamin B deficiency (Sullivan and Nicholls, '40a, b, '41, '42). There were changes in the luster of the hair. The coats were no longer sleek. The fur was bristly and uneven (fig. 8). In 25% of the animals there were areas of diffuse symmetric alopecia on the submental region (fig. 9), upper anterior venter, shoulders, occiput, in the preauricular regions and in the genital region. The onset of ocular symptoms and signs occurred at approximately the same time or soon after the cutaneous signs were remarked and differed from those described in experiment I in that, in some cases there was wide extension of the alopecia of the lids to the circumocular regions (fig. 10). In a few of the black and hooded rats greying was observed.

Microscopic examination

In the majority of the sections from rats in which deficiency signs had been apparent for 2 or 3 weeks, there were dis-

integration, dilatation and atrophy of the sebaceous glands. The epidermis was thin and there was a decrease in the thickness of the cutis with a loss of subcutaneous fat early in the stage of the disease (fig. 12). There was no disturbance of the connective tissue. In a few sections diffuse mononuclear cellular infiltration was observed in the cutis. In some sections the epidermis was wavy and there were a few patchy areas of acanthosis. Hyperkeratosis was observed in the majority of the sections, but it was much less extensive than that in experiment I. A few follicles were dilated and plugged with hyperkeratotic material. Again this was less extensive than the follicular dilatation and plugging of experiment I.

In summary, the microscopic findings were suggestive of some alterations which have been described in connection with the deficiencies of several members of the vitamin B complex, although there were, to a less extent, also the changes which characterized the sections from rats in experiment I which were considered to be deficient only in vitamin A. These findings indicated that 5% yeast was an insufficient source of the vitamin B complex under the experimental conditions and that many of the vague cutaneous signs previously attributed to vitamin A deficiency were due probably to complicating vitamin B deficiency.

In order to test this supposition further, the following additional individual supplements were fed thrice weekly to three groups of rats totalling twenty animals: thiamine, 50 μg.; pyridoxine, 50 μg.; pantothenic acid, 100 μg.; riboflavin, 40 μg., and nicotinic acid, 30 μg. Three similar control groups received the 5% yeast diet with no additional supplements. Retarded growth and the onset of cutaneous and ocular signs identical to those described for the 5% yeast level rats were remarked during the seventh and eighth weeks in the three control groups of "unfortified" litter mates. All of these animals died between the tenth and thirteenth weeks. By contrast there was a sustained increase in weight for 17 to 18 weeks in the three groups of rats that were fortified

with the crystalline B vitamins. These rats were lively and from all visible signs in good health. Their coats were sleek during the first 18 weeks of the experiment and there were no signs indicative of vitamin B deficiency. The two females in the group were inseminated four times by their male cage mates. In the first two pregnancies, normal sized litters were cast; these survived for 24 hours. There was a remarkably well-sustained, rapid growth. At 18 weeks the weights varied from 175 to 266 gm. There was, therefore, a 10-week delay in the onset of the ocular signs in the group receiving the additional crystalline B vitamins.

The onset of ocular symptoms and signs occurred from the eighteenth to the twentieth week. Thereafter the rats were weak and slightly humped. In many there were rales. The furs were dishevelled and infested with pediculi. When the infestations were first noted, the animals were 5 months old. Although the control group were not infested, it should be pointed out that they died before the third month of the experiment. There were no areas of alopecia, no dermatitis and no manifestations of achromotrichia. The vitamin A deficiency demonstrated by ocular and nasal signs progressed less rapidly than that observed in the control groups receiving 5% yeast. Food consumption decreased in the last few days of life. The mode of death and pattern of weight curve in the last 2 weeks were identical with those described in experiment I. There was rapid regrowth of shaved hair until the last few weeks of life. The microscopic findings were similar to those in experiment I. These findings demonstrated that: (1) the vitamin B deficiency signs which occurred in the animals reared on a 5% yeast diet were prevented by the addition of the crystalline factors, and (2) there was a delay in the appearance of vitamin A deficiency signs in the presence of adequate amounts of B vitamins and/or precipitation of the occurrence of vitamin A deficiency signs when the supply of B vitamins was inadequate, indicating a probable interrelationship. However, because one rat that had been reared on the "ideal" vita-

min A deficiency diet of experiment I survived for 27 weeks, the importance of the interrelationship was doubtful. Accordingly, four other groups of rats were carefully matched with litter mates to determine whether increased food consumption due to the extra vitamin B complex was operative. Also, groups of rats were restricted to the amount of food voluntarily selected by the A deficient rats receiving diet IIa, but they received vitamin A. This experiment was disappointing as it failed to confirm the initial experiment. The vitamin B fortification failed to delay the signs of vitamin A deficiency, except for 2 or 3 weeks in two of the groups. Also in two of the groups the "fortified" animals survived no longer than their "unfortified" litter mates. However, the question of diminished food intake was settled. In the rats receiving the restricted quantities of food, the only gross cutaneous abnormalities were slightly dishevelled hair and pediculosis when the animals were 5 months old; microscopic examination showed slight atrophy of the epidermis and appendages but no alterations suggestive of vitamin B deficiency or changes similar to those described in experiment I. Microscopic examination of the rats reared on the 5% yeast diet fortified with crystalline B vitamins showed changes identical to those described in experiment I.

B. Signs and symptoms produced by a diet containing 5% yeast as the source of the vitamin complex and hydrogenated cottonseed oil as the source of fat

Zimmerman and Cowgill ('36), Johnson ('39) and other investigators employed diets containing hydrogenated cottonseed oil as a source of fat and 5% or less yeast as the source of vitamin B. Therefore, four groups totalling twenty-four rats were fed the following diet: vitamin-free casein, 18%; sucrose, 61%; McCollum salts (no. 51), 6%; hydrogenated cottonseed oil,[1] 10%; yeast, 5%; viosterol, equivalent to 460 units of vitamin D, twice weekly. Choline chloride was not

[1] Crisco.

included in order that the diet might be identical with the experimental rations used by other investigators. The onset and severity of nasal and ocular signs were identical to those in group IIa. However, there were more extensive cutaneous changes, particularly diffuse symmetric areas of alopecia on the neck, shoulders and chest, which occurred in 90% of the rats. The microscopic findings resembled those in group IIa with the exception that the changes in the sebaceous glands were more extensive and hyperkeratosis and follicular plugging were less marked. These findings indicated that when supplemental choline was omitted and hydrogenated cottonseed oil was used in place of lard as the source of fat, there were more extensive complicating gross and microscopic cutaneous alterations and less evidence of the microscopic changes found in rats reared on a diet deficient only in vitamin A. Although the weight curves indicated that the control diet (fig. 1 f) was as nutritious as the control diet in experiment I, the rate of growth and the maximum weight attained (fig. 1g) by the deficient rats were 25% less than the rate of growth and the maximum weight attained by the deficient rats in experiment IIa.

EXPERIMENT III

THE INFLUENCE OF THE INADEQUACY OF FAT ON THE SIGNS OF VITAMIN A DEFICIENCY

The findings in experiments I and II indicated that the level of vitamin B affected the onset of the vitamin A deficiency signs and that an insufficient level of vitamin B complicated the vitamin A deficiency syndrome by producing superimposed signs of vitamin B deficiency. It was also apparent that the quality of fat in the deficiency rations was of importance. The sparing action of fat and fatty acids and the interrelationship with vitamin B deficiency is well known (Burr, '42). In order to test the effect of a fat-free diet on the signs of vitamin A deficiency, two groups of 21-day-old rats totalling sixteen animals and ranging in body weight from 25 to 40 gm. were fed the following diet: vitamin-free casein, 18%; sucrose, 71%; McCol-

lum salts (no. 51), 6%; yeast, 5%; viosterol twice weekly, vitamin E as alpha-tocopherol, 2.5 mg. twice weekly. The onset of nasal and ocular signs occurred 2 to 4 weeks sooner than in any group in which fat was incorporated into the diet. The ocular signs were severe. The onset of cutaneous signs immediately preceded the nasal and ocular signs. On the neck, shoulders and upper chest there were diffuse symmetric areas of alopecia unaccompanied by scaling. The patterns of alopecia resembled those observed in experiment IIa. Similar patterns of symmetric alopecia were observed in the control animals. All of the deficient rats died in 6 or 7 weeks. The control rats survived until they were sacrificed at 15 weeks. Generalized mild scaling was noted in the control rats after they were 8 weeks old. Microscopic examination of the deficient rats showed findings similar to those described in experiment II.

The pattern of the weight curve of the deficient animals (fig. 1i) was similar to those of experiment IIa and IIb. However, the animals failed to attain the maximum weight of those in IIa (fig. 1e) and slightly exceeded the maximum weight attained in IIb (fig. 1g).

EXPERIMENT IV

COMPLICATED SIGNS PRODUCED BY THE CURRENT UNITED STATES PHARMACOPOEIA VITAMIN A ASSAY DIET

The current United States Pharmacopoeia ('36) vitamin A assay diet is described as follows: casein, 18%; starch, 65%; salt mixture, 4%; vegetable oil, 5%; yeast, 8%; vitamin D suspended in vegetable oil, 3 U.S.P. units per gram carried by vegetable oil or yeast.

In view of the findings in experiments I, II and III, it was apparent that the United States Pharmacopoeia diet could be improved.

Two groups of young rats totalling sixteen animals and ranging in body weight from 30 to 40 gm. were fed the United States Pharmacopoeia vitamin A assay diet. For the first

4 weeks there was a gradual gain in weight (fig. 1 k) which approximated that of the control animals (fig. 1j). However, the weight gain was less than that achieved by the deficient animals in experiment I (fig. 1c). In the fifth and sixth weeks there was a plateau in the weight curve. After the fifth week, the control animals gained (fig. 1j) at a less rapid rate than the control animals in experiment I (fig. 1b). Death of the deficient rats occurred in from 7 to 8 weeks. Nasal and ocular signs of vitamin A deficiency were remarked between the fourth and fifth weeks. During the fifth week, diffuse areas of alopecia on the posterior aspect of the neck and on the shoulders were noted. Pattern greying occurred in the hooded and black rats (fig. 1i). Alopecia and pattern greying also were noted in the control rats. The growth curve of the control animals (fig. 1j) compared unfavorably with the growth curve of the control animals in experiment I. Microscopic examination disclosed findings similar to those described in experiment II.

The diet used in experiment I has the following advantages over the current United States Pharmacopoeia vitamin A assay diet: 10% lard was used instead of 5% vegetable fat. Yeast of a known assay was incorporated at a 10% level instead of yeast of an unspecified assay at an 8% level. Supplements of choline and recommended doses of vitamin E were additional improvements.

DISCUSSION

In the literature dealing with vitamin A deficiency of the rat, there are numerous reports of the pathologic alterations of various epithelial tissues (Wolbach and Bessey, '42). These include descriptions of changes in the eye, the paraocular glands, and the respiratory, gastrointestinal and genitourinary systems. The study of the skin of the vitamin A deficient rat has been neglected. The majority of the few recorded observations on this important epithelial tissue have been incidental to the more careful study of the eye and the paraocular

glands. McCollum, Orent-Keiles and Day ('39) describe the cutaneous changes as lesions "that appear when the animals are older than four months of age and that are in the form of scabby ears and tails, sores on the nose, sore feet and ragged hair." Recent reviews (Sullivan and Nicholls, '40 a; Eddy and Dalldorf, '41) also include this description. It was based on the observations of McCollum and his collaborators of thousands of rats subsisting on experimental rations which, in the light of present knowledge, must now be considered to have been deficient in some other essential aliments as well as in vitamin A. In the first, second, third and fourth editions of McCollum's ('18, '22, '25, '29) texts photographs of vitamin A deficient rats exhibit cutaneous signs which indicate that the animals probably suffered also from vitamin B deficiency. Also, inasmuch as the cutaneous signs usually appeared when the animals were older than 4 months, the question of complicating fat deficiency must be considered.

In rats receiving a diet adequate in all essential aliments save vitamin A, there was found microscopic evidence of a process somewhat resembling the cutaneous changes described in Chinese soldiers by Frazier and Hu ('31, '36) and attributed to vitamin A deficiency. When the vitamin A deficient diet contained an inadequate amount of a natural source of vitamin B there were complicating gross and microscopic findings which altered the picture of the lesion that is apparently the result of vitamin A deficiency. Absence of fat in the vitamin A deficient diet and the quality of fat that was present also influenced the onset and severity of complicating cutaneous changes. The necessity of the inclusion of vitamin E in the vitamin A deficient diet is readily appreciated by us although data yielded by these experiments add no conclusive proof of its indispensability. Mason ('30) showed that in vitamin A deficiency the requirement for vitamin E was increased.

The findings in experiment II indicated that (1) when the level of yeast was 5% and the source of fat was supplied by

a vegetable oil, there was a high incidence of complicating gross and microscopic signs of vitamin B deficiency; and (2) when lard was used in place of hydrogenated cottonseed oil, and the yeast level was 5%, the incidence of complicating signs of B deficiency was decreased although not prevented.

The findings in experiment III demonstrated some features of the complex interrelationship of deficiencies of vitamin A, vitamin B and fat, and clearly proved that diets used for vitamin A assay or the study of vitamin A deficiency signs should contain adequate fat and essential unsaturated fatty acids.

The findings in experiment IV demonstrated that in rats reared on the current United States Pharmacopoeia vitamin A assay diet the signs of vitamin A deficiency were complicated. The quality of fat apparently is of importance in the interrelationship of vitamins A and B deficiencies. Therefore, the use of lard probably is more desirable than the use of a vegetable oil in an official assay diet. Also the designation "vegetable oil" should be qualified. Certain vegetable oils, for example, avocado oil, contain vitamin A (Weatherby, '30). Yeast of a known potency should be used. In the present experiment 10% rather than 5% yeast was found to promote better growth in the deficient rats as well as in the control animals, and it was found also that the growth rate of rats maintained on a diet containing 5% yeast could be improved by the addition of the crystalline factors of the vitamin B complex. At least 10% of a potent yeast of known assay containing amounts of the vitamin B complex equivalent to recommended necessary crystalline vitamin B supplements should be employed in vitamin A assay diets. Supplementation with choline and vitamin E also contributes to the refinements calculated to achieve an experimental ration deficient in one vitamin.

SUMMARY AND CONCLUSIONS

1. When young rats were fed a diet deficient only in vitamin A insofar as it is now possible to concoct such a ration, there resulted a deficiency disease, the gross cutaneous signs

of which differed from the previously accepted descriptions of vitamin A deficiency.

2. Complicating deficiencies of the vitamin B complex, fat and essential fatty acids were responsible for cutaneous alterations which resulted in the misrepresentation of the vitamin A deficiency syndrome.

3. The data obtained in this study indicate that the diets used hitherto for producing vitamin A deficiency in rats and the current United States Pharmacopoeia vitamin A assay diet should be revised when the objective is to obtain the picture of the uncomplicated deficiency.

LITERATURE CITED

BURR, G. O. 1942 Significance of the essential fatty acids. Fed. Proc., vol. 1, p. 224.

EDDY, W. H., AND G. DALLDORF 1941 The Avitaminoses, 2nd ed. The Williams and Wilkins Company, Baltimore.

FRAZIER, C. N., AND C. K. HU 1931 Cutaneous lesions associated with a deficiency in vitamin A in man. Arch. Int. Med., vol. 48, p. 507.

———— 1936 Nature and distribution according to age of cutaneous manifestations of vitamin A deficiency. Arch. Dermat. and Syph., vol. 33, p. 825.

IRVING, J. T., AND M. B. RICHARDS 1938 Early lesions of vitamin A deficiency. J. Physiol., vol. 94, p. 307.

JOHNSON, M. L. 1939 The effect of vitamin A deficiency upon the retina of the rat. J. Exp. Zool., vol. 81, p. 67.

MASON, K. E. 1930 The specificity of vitamin E for the testes. I. Relation between vitamins A and E. J. Exp. Zool., vol. 55, p. 101.

MCCOLLUM, E. V. 1918 The Newer Knowledge of Nutrition. The Macmillan Company, New York, p. 88.

———— 1922 The Newer Knowledge of Nutrition. The Macmillan Company, New York, p. 38.

———— 1929 The Newer Knowledge of Nutrition. The Macmillan Company, New York, p. 164.

MCCOLLUM, E. V., E. ORENT-KEILES AND H. G. DAY 1939 The Newer Knowledge of Nutrition, 5th ed. The Macmillan Company, New York, p. 310.

MCCOLLUM, E. V., AND N. SIMMONDS 1925 The Newer Knowledge of Nutrition. The Macmillan Company, New York, p. 285.

SULLIVAN, M., AND J. NICHOLLS 1940 a The nutritional approach to experimental dermatology. Introduction and review of the literature. J. Invest. Dermat., vol. 3, p. 309.

———— 1940 b Nutritional dermatoses in the rat. I. Vitamin B_6 deficiency. J. Invest. Dermat., vol. 3, p. 317.

———— 1941 Nutritional dermatoses in the rat. IV. Riboflavin deficiency. J. Invest. Dermat., vol. 4, p. 181.

———— 1942 Nutritional dermatoses in the rat. VI. The effect of pantothenic acid deficiency. Arch. Dermat. and Syph., vol. 45, p. 917.

THE PHARMACOPOEIA OF THE UNITED STATES. XI. 1936 Decennial Revision. Mack Printing Company, Easton, Pa., p. 479.

WEATHERBY, L. 1930 Experiment with avocado oil. Year Book, California Avocado Association, p. 105.

WOLBACH, S. B., AND O. A. BESSEY 1942 Tissue changes in vitamin deficiencies. J. Nutrition, vol. 22, p. 233.

ZIMMERMAN, H. M., AND G. R. COWGILL 1936 Lesions of the nervous system in vitamin deficiency. J. Nutrition, vol. 11, p. 411.

PLATE 1

EXPLANATION OF FIGURES

2 Showing general appearance of vitamin A deficient rat reared on a diet containing 10% yeast, 10% lard and supplemented with choline and vitamin E.

3 Showing early ocular signs of deficiency. Edema and alopecia are limited to the lids. The corneal opacity is in the early stage. There is no extension of the alopecia of the lids to the circumjacent region.

4 Section of back × 60 (exp. I). Showing hyperkeratosis, dilatation of upper portion of hair follicle and preservation of the lower half of the follicle. The dilated space has been "plugged" with hyperkeratotic lamellae.

5 Section from tail × 60 (exp. I). Showing dilatation of one entire follicle and "plugging" by the product of hyperkeratosis.

6 Section from tail × 60 (exp. I). Note the hyperkeratosis and lamellae arranged in concentric whorls around the hairs.

7 Section from tongue × 60 (exp. I). Showing large epithelial-lined spaces containing hyperkeratotic lamellae (keratinizing metaplasia). Also note the extensive cellular infiltration and the abscess.

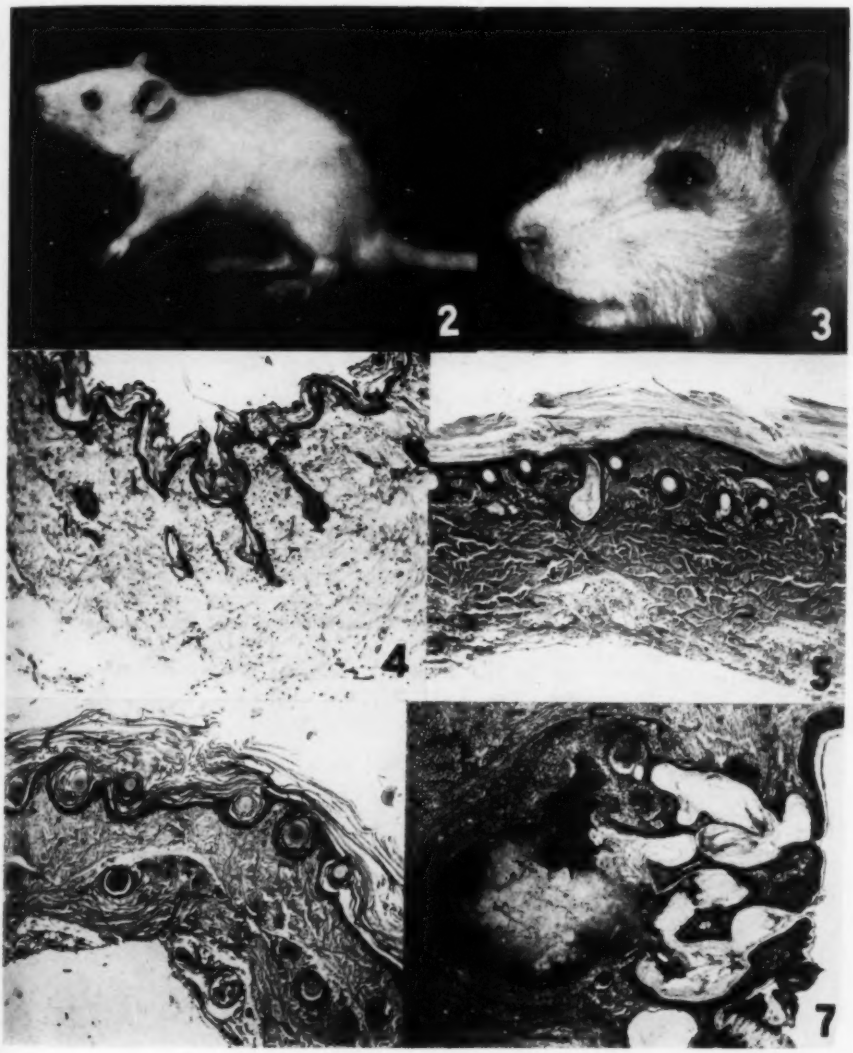
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PLATE 2

EXPLANATION OF FIGURES

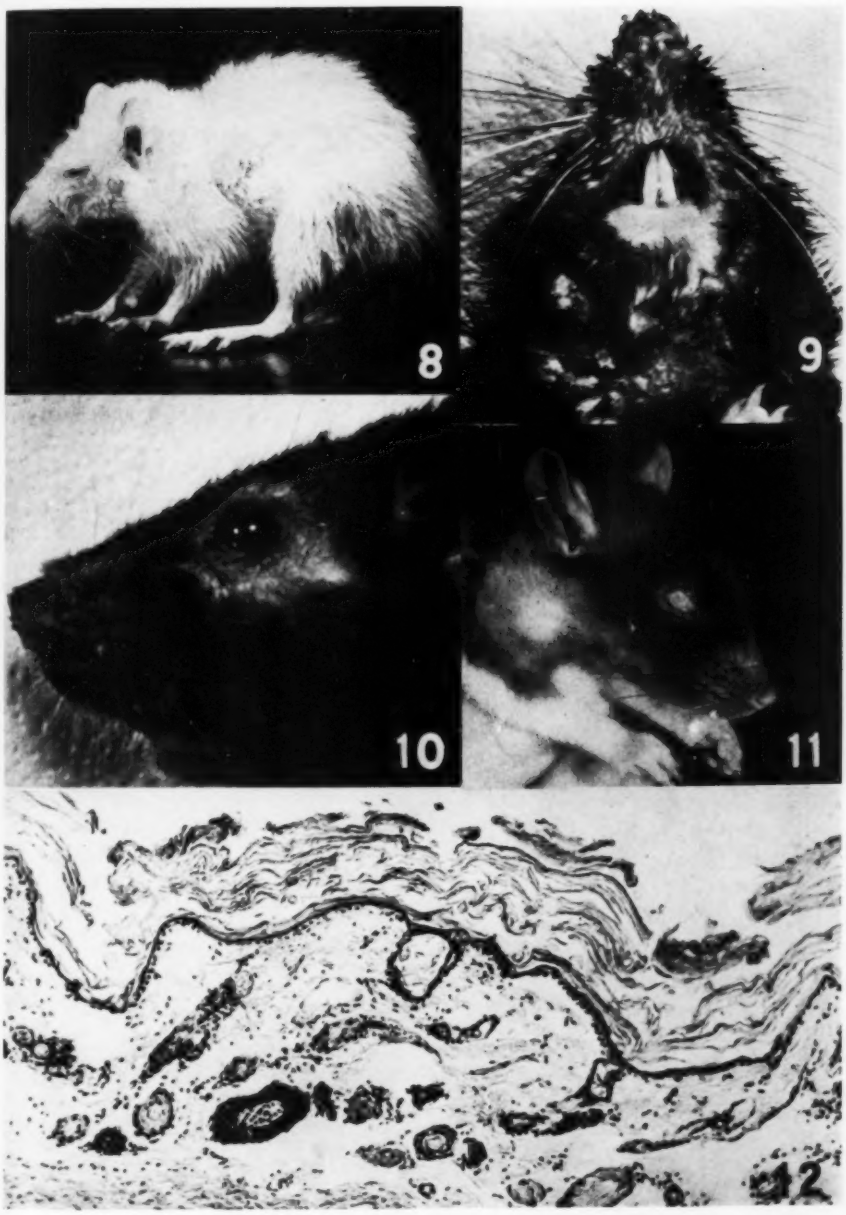
8 Showing signs suggestive of vitamin B complex deficiency in vitamin A deficient rats reared on a diet containing 5% yeast. Note the ungroomed ragged dishevelled hair, the humped posture and the general poor condition. Contrast with figure 2.

9 Alopecia and dermatitis in submental region (exp. II).

10 Widespread circumocular alopecia in rat reared on 5% yeast (exp. II).

11 Vitamin A deficiency produced by the United States Pharmacopoeia vitamin A assay diet. Note the complicating cutaneous signs of vitamin B complex deficiency, namely, symmetric alopecia on shoulders and side of snout, and symmetric graying.

12 Section from back × 180 (exp. II). Showing atrophy of epidermis, sebaceous glands and cutis and mild diffuse cellular infiltration.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NICOTINIC ACID STORAGE IN THE DOG AT DIFFERENT DOSE LEVELS OF THE VITAMIN [1]

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THREE FIGURES

(Received for publication October 31, 1942)

The present study was designed to determine whether in dogs subjected to repeated attacks of blacktongue, and cured by nicotinic acid (niacin) or its amide, the periods of protection against subsequent experimentally induced attacks are prolonged by successive increases in the dose of the curative drugs.

EXPERIMENTAL

The study was conducted with ten mongrel adult dogs subjected to 140 attacks of blacktongue over a period of 2 years, beginning September, 1940. The animals were placed on the following experimental blacktongue producing diet of natural foodstuffs, low in most of the B vitamins, but particularly low in nicotinic acid (niacin): yellow corn meal 60.0%, cow peas 9, casein 14.5, sucrose 4.8, cottonseed oil [2] 4.8, cod liver oil 4.8 and salt mixture (having a percentage composition of sodium chloride 52.6, magnesium citrate 21.0, calcium lactate 21.0, and iron citrate 5.4, with 20 cc. of Lugol's solution added) 2.1%. The corn meal, peas and salt mixture were mixed with three volumes of water and cooked for 2 hours in an open steam cooker. After cooling, the other ingredients were added

[1] Part of the expense of this investigation was met by a grant from the Lederle Laboratories, Pearl River, N. Y.

[2] Wesson.

and the whole thoroughly mixed. This diet was supplemented with 10 mg. of thiamine and 2 mg. of riboflavin per dog per week.

The assay method used was based on that of Harvey, Smith, Persons and Burns ('38), with certain significant changes. The diet used in the present study was almost identical with that used by these observers, and the 10-day treatment regime remained the same, but there are three important differences in the method used in this study.

1. Much more care was taken in the selection of the animals; only dogs having light-colored pale pink mouths, in which the early signs of blacktongue could easily be recognized, were employed. The same dog was used repeatedly at different dose levels, thus serving as his own control.

2. To make this feasible each therapeutic test period was followed by a regenerative period in which the dog was given 5 mg. of nicotinic acid and 10 gm. of dry brewers' yeast for the 10-day dosing period, then allowed to develop blacktongue, at which time another therapeutic test was made. This was done with the idea of maintaining a constant level of the other members of the B complex which have been shown to be necessary for consistent results with nicotinic acid therapy (Margolis, Margolis and Smith, '39; Smith, '40).

3. In the present study much emphasis has been placed upon the accurate delimitation of the periods of the tests. The first symptom observed in the deficient dogs was a slight but definite reddening of the inside of the upper lip; this is designated a one plus (+) mouth lesion, and can be detected only in animals with suitable light-colored mouths. The second stage in the development of mouth lesions shows a more extensive, well-defined deep red area on the inside of the upper lip, and this has been designated two plus (++) (fig. 1 A). At this stage there is usually some excess salivation. A three plus (+++) mouth lesion exhibits a still larger red area and there is always excessive salivation. A four plus (++++) mouth lesion is characterized by a diffuse deep red color with beginning gangrene about the margin of the teeth and large

amounts of foul-smelling saliva. At this stage, which follows closely after the +++ mouth lesion, there is always marked loss of weight and recovery is very uncertain. Consequently the ++ mouth lesion is the safest endpoint for delimiting the test period in this type of assay.

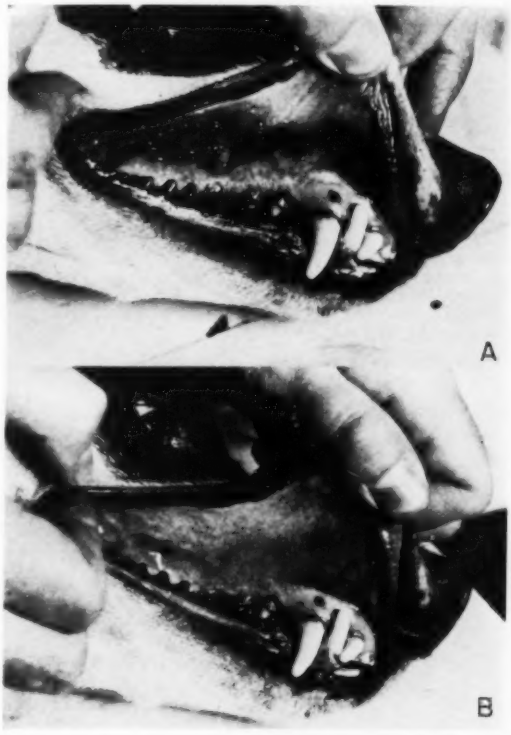

Fig. 1A Dog no. 112 with a ++ blacktongue mouth lesion. 1B, same dog with a negative mouth after 10 days' treatment with nicotinic acid (10 mg. per kilo per day).

The test period selected begins with an animal showing a ++ mouth lesion. A therapeutic dose of nicotinic acid, or its amide, is then given for 10 days only, the dog remaining all the while on the deficient diet. The mouth lesion heals, and when

the dog again shows a ++ mouth lesion the test period is ended. The interval of time from the cessation of dosing at the end of 10 days to the development of a ++ mouth lesion is designated the "storage protective period."

A comparison was made of the biological effect of nicotinic acid and nicotinic acid amide fed at the following dose levels: 0.1 mg., 0.2 mg., 0.3 mg., 0.4 mg., 0.5 mg., 1.0 mg., 5.0 mg.,

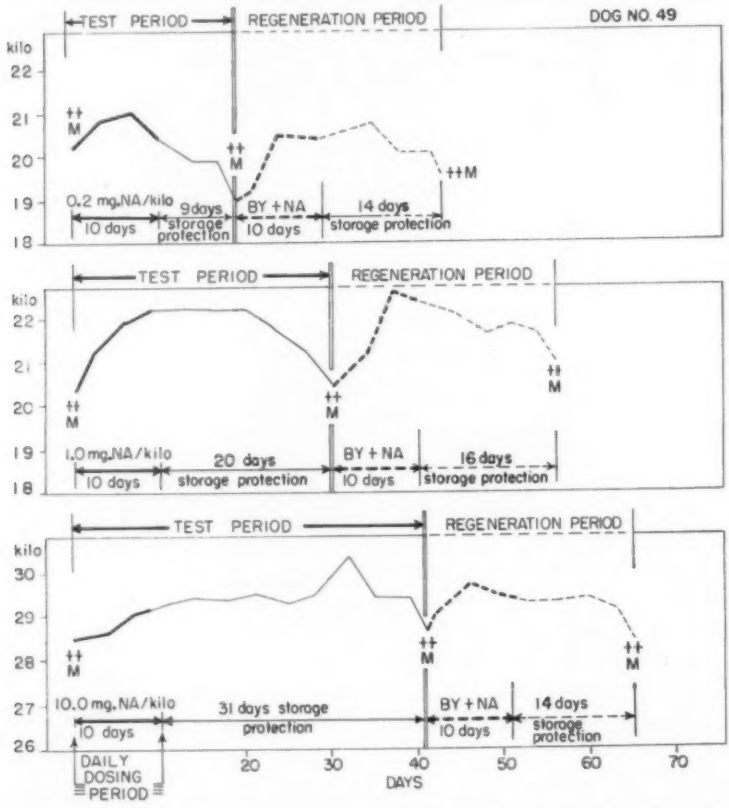

Fig. 2 Illustrates the performance of dog no. 49 at the following three dose levels of nicotinic acid: 0.2, 1.0 and 10 mg. per kilo per day. M indicates a ++ mouth lesion. During the regeneration period the dog received 5 mg. nicotinic acid and 10 gm. of dry brewers' yeast per day for the 10-day period.

10.0 mg. and 20.0 mg. per kilo of body weight. The smaller doses, 1 mg. and below, were given parenterally, and the larger ones orally, since it has been demonstrated that at a level of 1.5 mg. per kilo there is no significant difference in results between oral and parenteral therapy (Margolis, Margolis and Smith, '38). All doses were given daily for a period of 10 days and then discontinued.

RESULTS

There is little or no difference in the biological effect of nicotinic acid and nicotinic acid amide.

The dose level of 0.1 mg. per kilo was entirely inadequate; the mouth never became normal. At the level of 0.2 mg. per kilo the mouth returned slowly to normal, but this level was not entirely satisfactory as a curative dose because of a delayed reaction. At 0.3 mg. per kilo the cure is prompt although the protective period is approximately the same as with 0.2 mg. Table 1 indicates the increased protection due to the increased dose levels.

TABLE 1

The storage protective values of graded doses of nicotinic acid and nicotinamide in canine blacktongue.[1]

DOSE PER KILO	NUMBER OF TESTS			STORAGE PROTECTIVE PERIOD		
	With nicotinic acid amide	With nicotinic acid	Total	Nicotinic acid amide	Nicotinic acid	Average
0.1	6		6	4.5		4.5
0.2	6	4	10	8.3	7.3	7.9
0.3	5	5	10	7.0	9.0	8.0
0.4	4	4	8	9.5	10.7	10.1
0.5	4	4	8	13.5	13.0	13.3
1.0	4	3	7	19.0	21.7	20.1
5.0	3	2	5	30.3	31.5	30.8
10.0	3	6	9	42.3	28.5	33.1
20.0	3	1	4	36.3	35.0	36.0

[1] The average value obtained from fifty-one tests for the storage protection during the regeneration interval between tests when the dogs received 10 gm. brewers' yeast and 5 mg. nicotinic acid was 16 days.

There is increased protection from a subsequent attack of blacktongue with an increase in dose level of nicotinic acid or nicotinic acid amide up to 10 mg. per kilo. This increase is a function of the logarithm of the dose (fig. 3).

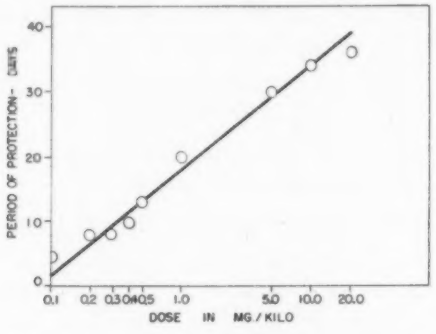

Fig. 3 Average storage protection afforded by nicotinic acid and nicotinic acid amide at different dose levels plotted logarithmically.

DISCUSSION

In a study such as this the selection of a very definite but conservative endpoint in determining the test and protective period is important; hence, our choice of a ++ mouth lesion which is quite red, easy to identify, and yet not too far advanced. If the deficiency goes beyond this stage we reach a period in the syndrome characterized by complete anorexia and precipitous weight loss. This is due to a complete nutritional breakdown, it would seem, rather than to the particular deficiency under consideration.

The type of storage observed in these experiments was not seen in our earlier work or in that of other investigators (Margolis, Margolis and Smith, '38; Birch, '39; Schaefer, McKibbin and Elvehjem, '42). This is explained by the difference in methods, the present one being designed specifically for determining degrees of nicotinic acid storage under varying dosage, other factors being constant.

The relapses and remissions of human pellagra can best be explained on the basis of a definite but limited body storage of the protective factor, nicotinic acid or its physiologically active derivatives.

SUMMARY AND CONCLUSIONS

Ten mongrel adult dogs have been studied in 140 attacks of blacktongue over a period of 2 years. Test doses of nicotinic acid and nicotinic acid amide have been given for periods of 10 days to dogs subsisting on a blacktongue producing diet of natural foodstuffs and the period of protection against subsequent attacks observed at the following daily dose levels: 0.1 mg., 0.2 mg., 0.3 mg., 0.4 mg., 0.5 mg., 1.0 mg., 5.0 mg., 10 mg. and 20 mg. per kilo of body weight. An increased protection was observed with increase in dose level. The increase in protection was not, however, proportional to the increase in dose level but was a function of the logarithm of the dose.

These results indicate a limited, but definite storage of nicotinic acid, or one of its physiological derivatives in the body of the dog.

ACKNOWLEDGMENT

We wish to thank Thomas E. Lasater for his very excellent care of the animals and other technical assistance.

LITERATURE CITED

BIRCH, T. W. 1939 The requirements of the dog and the rat for nicotinic acid. J. Nutrition, vol. 17, p. 281.

HARVEY, H. I., D. T. SMITH, E. L. PERSONS AND M. V. BURNS 1938 The effect of various fractions of liver on experimental canine blacktongue. J. Nutrition, vol. 16, p. 153.

MARGOLIS, G., L. H. MARGOLIS AND S. G. SMITH 1938 A cure of experimental canine blacktongue with optimal and minimal doses of nicotinic acid. J. Nutrition, vol. 16, p. 541.

MARGOLIS, L. H., G. MARGOLIS AND S. G. SMITH 1939 Secondary deficiency of vitamin B_1 and riboflavin in the blacktongue producing diet. J. Nutrition, vol. 17, p. 63.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Nicotinic acid deficiency studies in dogs. J. Biol. Chem., vol. 144, p. 679.

SMITH, S. G. 1940 The importance of recognizing secondary vitamin deficiencies. Am. J. Trop. Med., vol. 20, p. 593.

THE AVAILABILITY FOR HUMAN NUTRITION OF THE VITAMIN C IN RAW CABBAGE AND HOME-CANNED TOMATO JUICE

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ONE FIGURE

(Received for publication November 3, 1942)

Very few experiments have been carried out to determine the comparative availability for human nutrition of a natural food source of vitamin C and the pure vitamin. Todhunter and Fatzer ('40) studied the utilization of the vitamin in red raspberries, and Clayton and Folsom ('40) that in baked potatoes. Hawley, Stephens and Anderson ('36) determined the proportion of the vitamin excreted in the urine when it was given in equivalent amounts as crystalline ascorbic acid and orange juice. In all of these studies the conclusion was reached that the vitamin C in the test food was as well utilized as that in crystalline form. An examination of the data of Hawley and associates shows, however, that there was considerable variation in the response of the various subjects. In an experiment on only one subject Goddard and Preston ('38) noted a higher percentage output of vitamin C on a base forming diet containing orange juice than on a diet containing the same amount of base plus crystalline ascorbic acid. It therefore appears that the question of the comparative availability of crystalline ascorbic acid and the vitamin C of orange juice has not yet been definitely answered and that the availability of the vitamin may be related to the availability of the base of the food in which it occurs, or to some other factor.

The purpose of the present experiment was to study the utilization of the vitamin C in raw cabbage and home-canned tomato juice. There appeared to be a possibility that, at least in some subjects, some of the vitamin of raw cabbage might be lost in digestion and that excess base from tomato juice might affect the utilization of the vitamin.

In the United States these two foods are used as important sources of vitamin C, especially in those localities where citrus fruits are expensive or not available. Cabbages can be raised in all parts of Maine, but in previous years farmers in the northern part of the state have had considerable difficulty in getting tomatoes to ripen, owing to the short growing season.[1]

As shown by E. F. Murphy ('42), the amount of vitamin C in any one variety of cabbage or tomato may vary considerably according to locality and weather conditions, but under identical conditions some varieties are much higher than others. Of early varieties of cabbage grown in Maine, Murphy [2] has found Cornell Early and Jersey Wakefield to be high in vitamin C. The Ballheads, which come later, are also high.

EXPERIMENTAL

The method used in the present study is similar to the one used in a previous study on the potato (Clayton and Folsom, '40), with the few changes noted herein. The subjects were four college students (three men and one woman), each of whom ate the same basal diet (containing approximately 10 mg. vitamin C) throughout the experiment. To this was added pure vitamin C, or the food to be tested plus pure vitamin C. Slight adjustments were made in the diet of each subject to allow for caloric requirement and individual preference, but in each case the diet was kept potentially

[1] Recently, however, Iva M. Burgess of the Maine Agricultural Experiment Station, has developed an early variety called Farthest North C, which will mature in August if planted early indoors and set out as soon as danger of frost is past. This variety is especially high in vitamin C, the values for the raw fruit ordinarily ranging from 29 to 35 mg. per 100 gm. The analyses were carried out by M. M. Monroe.

[2] Personal communication.

neutral in reaction. This was done in order to show any possible effect on vitamin C retention of the potential acidity or alkalinity of the test food added to it. The basal diet of subject I.H. is given in table 1.

The experiment was carried out in February, March, and April, 1942, and cabbage was the first food to be tested. The cabbages were of the Danish Ballhead variety and were raised in Maine. They were obtained from a commercial cold storage plant and showed fairly high initial vitamin C values (average 47.6 mg. per 100 gm.). However, after being kept for about a week in an electric refrigerator in the laboratory the values fell as low as 23 mg. It was then necessary to secure another lot to finish the experiment. These averaged 53.9 mg. per 100 gm.

When cabbage was being fed it was eaten at dinner in place of the green beans and raw carrots of the basal diet. This substitution changed the calculated acid base values of the diet very slightly, making it faintly acidic, even though cabbage itself is potentially basic. The amount to be eaten daily was determined by analysis [3] of each individual head. The portions were eaten plain, immediately after cutting from the head. Table 2 gives the analyses of the heads used and the amounts eaten each day to supply 50 mg. of vitamin C.

The tomato juice [4] was made by removing stems and imperfections from about 10 pounds of tomatoes, cutting them in pieces, and cooking in an aluminum kettle until just soft. They were then quickly strained in an aluminum cone-shaped strainer, using a wooden cone-shaped pestle. After being

[3] Both the reduced and dehydro forms of the vitamin were determined. Extraction was made with 3% metaphosphoric acid and the extracts titrated with dichlorophenolindophenol before and after H_2S reduction. Before reduction the pH was adjusted to 3.5 with citrate buffer according to the method of Bessey ('38). Ten milliliters of the extract and the required amount of buffer (about 4 ml.) were measured into 50 ml. graduated cylinders (without lip). H_2S was run in for 10 minutes and the extracts were stoppered and allowed to stand 30 minutes. The H_2S was then removed with a stream of wet CO_2, using an open system.

[4] The Farthest North C tomatoes used in making the juice for this experiment were raised at Highmoor Farm by Iva M. Burgess.

TABLE 1

Basal diet for subject I.H.

MATERIALS	MEASURE	WEIGHT	VITAMIN C[1]	EXCESS ACID [2]	EXCESS BASE [2]
		gm.	*mg.*	*cc. N sol.*	*cc. N sol.*
B complex capsule	1		—		
Apricot juice	½ c.	137	.96	—	4.82
Shredded wheat	1½ biscuits	42	—	5.10	—
Milk, pasteurized	1 c.	244	.30	—	4.37
Cream, pasteurized (40%)	⅛ c.	83	—	—	.33
Bread, whole wheat	2 slices	60	—	4.40	—
Butter	1 tb.	13	—	—	—
Coffee	1½ c.	355	—		
Sugar	1 tb.	13	—	—	—
Orange marmalade	1 tb.	22	.92	—	.22
Soup: milk	1 c.	244	—	—	4.37
Soup: celery	1½ c. raw	62	.25	—	4.84
Soup: flour	¾ tb.	7	—	.70	—
Soup: butter	½ tb.	7	—	—	—
Butter	1½ tb.	20	—	—	—
Bread, whole wheat	3 slices	90	—	6.60	—
Cheese, American	1 inch cube	20	—	1.10	—
Dates	8	58	—	—	6.38
Egg, E.P.	1	50	—	5.50	—
Apple, McIntosh	1 small	100	1.50	—	3.70
Beef round	3 cakes	170	—	18.02	—
Brown rice	4½ tb.	57	—	5.30	—
Carrot, raw	4 small strips	40	1.68	—	5.40
Green beans, canned	½ c.	66	2.84	—	3.10
Pears, canned	2 halves	95	1.05	—	3.40
	2 tb. juice	36 cc.	.50	—	
Graham crackers	2	19	—	1.52	—
Butter	1 tb.	13	—	—	—
Cocoa (1 c. milk)	1½ c.	274	—	—	4.37
Dates	2	15	—	—	1.65
Totals			10.00	48.24	46.95

Nutritive value of above diet

Calories	3227	Iron	22.9 mg.
Protein	105.4 gm.	Vitamin A	10,680 I.U.
Calcium	1.50 gm.	Vitamin B_1	2.9 mg.
Phosphorus	2.16 gm.	Riboflavin	2.5+ μg.
		Niacin	21.7+ mg.

[1] Reduced plus dehydro forms by analysis. Dashes indicate that vitamin C was assumed to be absent.

[2] Blank spaces indicate no data available.

TABLE 2

Vitamin C analyses of cabbages and tomato juice used.

Date 1942	CABBAGES			Date 1942	TOMATO JUICE		
	Reduced form	Reduced plus dehydro forms	Amount eaten for 50 mg. vitamin C		Reduced form		Amount eaten for 50 mg. vitamin C
					Juice only	Juice and pulp	
	mg./ 100 gm.	*mg./ 100 gm.*	*gm.*		*mg./ 100 ml.*	*mg./ 100 ml.*	*ml.*
3/6	42.36	47.28	114	4/14	17.20	No analysis of pulp	291 [1]
3/7	40.82	48.15	112				
3/8	42.35	47.43	114	4/15	14.85	18.50	270
3/9	32.98	36.34	148	4/16	20.40	26.65	188
3/10	35.24	41.64	129	4/17	17.30	22.50	222
3/11	35.24	41.64	129	4/18	20.40	26.70	187
3/12 [2]	47.74	55.83	97	4/19	17.80	23.40	214
3/13	50.70	54.57	99	4/20	21.40	34.30 [3]	146
3/14	47.86	51.19	105	4/21	16.30	22.50	222
				4/22	18.06	23.19	216
	Average	47.80	116		Average	24.72	208 [4]

[1] Based on juice only.
[2] New lot.
[3] High amount of pulp.
[4] Amount for 4/14 omitted.

quickly brought to the boiling point the juice was poured into sterilized quart jars and sealed. One teaspoon of salt was put into the bottom of each jar before the juice was added.

When tomato juice was being fed, the apricot juice of the basal diet was omitted and 150 ml. of the tomato juice was taken at breakfast. The remainder of the amount necessary to supply 50 mg. of the vitamin was taken at dinner. This substitution made the diet slightly basic. Table 2 gives the daily analyses [5] of the juice and the amounts eaten.

As in the potato study, the utilization of the vitamin C in the test foods was judged from the results of both blood and urine analyses. In the present experiment, however, the subjects were saturated before each test period. For 8 days

[5] Only the reduced form of the vitamin was determined, since the juice was found to contain none of the dehydro form.

before the experiment began the subjects were on their ordinary diets, but took 150 mg. of extra vitamin C daily (75 mg. at breakfast and 75 mg. at lunch, in the form of 25 mg. tablets). The actual experiment lasted 51 days and was divided into six periods as follows:

Period 1 (Saturation). Duration 7 days. The subjects were on the basal diet plus 200 mg. of vitamin C daily (one 100 mg. tablet at breakfast and another at lunch). Twenty-four-hour urine specimens were collected daily in liter-size amber glass bottles. The preservative used was 20 gm. of metaphosphoric acid and 20 ml. of glacial acetic acid to each bottle. The amount of vitamin C in the urine was determined daily by indophenol titration.

Period 2 (Vitamin C tablets). Duration 9 days. The subjects were on the basal diet plus 75 mg. of vitamin C daily (three 25 mg. tablets at breakfast). The purpose of this period was to determine to what extent saturation could be maintained by 75 mg. of the pure vitamin. This is the amount recommended by the Committee on Foods and Nutrition of the National Research Council for a 70-kg. man. (The subjects actually received 85 mg., counting the 10 mg. in the basal diet.) Blood samples were taken before breakfast on the first day (results belong to period 1). The samples of blood plasma were tested for vitamin C by a modification [6] of the Ingalls ('37) macro method. The results of the blood tests, together with the urine figures for the previous day, gave the saturation levels for the four subjects, with which the results for period 2 could be compared. For the first 8 days the urine preservative was the same as in period 1 and vitamin C analyses were made daily. On the ninth day, 25 ml. of toluene and 25 ml. of mineral oil were added to each bottle. The pH of the urine for this day was determined by the quinhydrone electrode method, but the vitamin C analysis was omitted.

Period 3 (Saturation). Duration 9 days. Same as period 1, except that blood samples were taken before breakfast on the morning of the first day (results belong to period 2).

[6] No potassium cyanide was used to preserve the vitamin C, as was suggested in the original method.

Period 4 (Cabbage). Duration 9 days. The subjects were on the basal diet (modified as previously stated), plus 25 mg. vitamin C (1 tablet at breakfast), plus the amount of raw cabbage needed to provide 50 mg. of vitamin C. The purpose of period 4 was to determine to what extent the vitamin C of the cabbage, supplemented by the 25 mg. in tablet form, would maintain saturation and how the results would compare with those secured in periods 2 and 6. Blood samples were taken before breakfast on the first day and tested for vitamin C (results belong to period 3). Urine samples were collected and vitamin C and pH tests made as in period 2. Fasting blood samples were also taken on the last day of this period. Between the end of this period, and the beginning of the next, there was an interval of 22 days. For 6 days before the beginning of period 5 the subjects were on their usual diets plus 150 mg. of vitamin C.

Period 5 (Saturation). Duration 8 days. Same as period 1.

Period 6 (Tomato juice). Duration 9 days. The subjects were on the basal diet (modified as previously stated) plus 25 mg. vitamin C (1 tablet at breakfast), plus the amount of tomato juice needed to provide 50 mg. of vitamin C. The purpose of period 6 was to determine to what extent the vitamin C of the tomato juice, supplemented by 25 mg. in tablet form, would maintain saturation and how the results would compare with those secured in periods 2 and 4. Fasting blood samples were taken on the first and last days and tested for vitamin C (results for first day belong to period 5). Urine samples were collected and vitamin C and pH tests made as in period 4.

RESULTS AND DISCUSSION

The results of the experiment are shown in figure 1 and tables 3 and 4. It will be noted from figure 1 that the excretion of vitamin C was fairly constant on the last 5 days of each period. Therefore, as an aid in evaluating the results, the average daily excretion for these 5 days in each period was determined for each subject as shown in table 4. This table shows that, judging from the average excretion of vitamin C in the urine during the last 5 days of each saturation period,

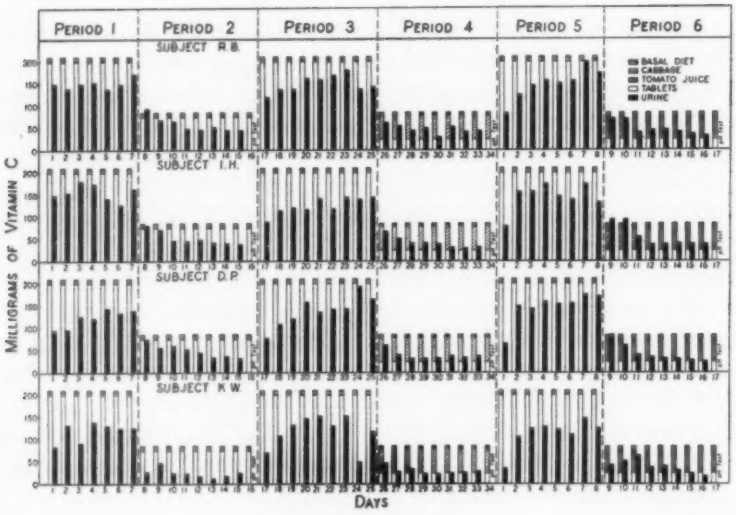

Figure 1

all of the subjects were completely saturated at the beginning of periods 2, 4, and 6. (More than 50% of the intake of 200 mg. of vitamin C was excreted.) With the exception of the results for two subjects in period 5, the blood figures during the saturation periods were above 1 mg. per cent.

Before comparing the results for the three test periods, attention is called to the fact that throughout the experiment,

TABLE 3

Data regarding subjects and results of determinations of vitamin C in blood plasma.

SUBJECTS[1]	HEIGHT	Weight[2]	BLOOD VITAMIN C (MG. PER 100 ML. IN PERIODS)					
			1 Saturation	2 Vit. C tablets	3 Saturation	4 Cabbage	5 Saturation	6 Tomato
	inches	*pounds*						
R.B. (F22)	63	127–128	1.25	.86	1.11	1.18	1.26	.86
I.H. (M22)	72	172–169	1.25	.78	1.03	.78	.94	.78
D.P. (M21)	70	171–168	1.09	.54	1.03	.86	.94	.93
K.W. (M20)	68	135–141	1.25	.93	1.26	.94	1.26	.93

[1] Data in parentheses indicate sex and age.

[2] Weight beginning and end of experiment.

TABLE 4

Average amount of vitamin C excreted and percentage of intake excreted in the urine on last 5 days of each period; pH of the urine.

SUBJECTS	PERIOD						URINE pH IN PERIODS		
	1 Saturation	2 Vit. C tablets	3 Saturation	4 Cabbage	5 Saturation	6 Tomato	2 Vit. C tablets	4 Cabbage	6 Tomato
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
R.B.	152.1	47.8	157.8	43.3	167.2	40.2	6.5	6.2	6.4
	(72.4)[1]	(56.2)	(75.2)	(51.0)	(79.6)	(47.9)			
I.H.	157.2	43.3	138.6	35.0	154.3	39.2	5.9	6.5	5.9
	(74.9)	(50.9)	(66.0)	(41.2)	(73.5)	(46.7)			
D.P.	132.9	40.5	155.9	35.2	163.6	30.9	6.0	6.5	5.9
	(63.3)	(47.7)	(74.3)	(41.4)	(77.9)	(36.8)			
K.W.	121.9	19.4	138.6[2]	25.0	127.6	30.1	6.0	5.2	5.9
	(58.1)	(22.8)	(66.0)	(29.4)	(60.8)	(35.9)			

[1] Figures in parentheses indicate percentage of intake.
[2] One day omitted in average — specimen incomplete.

subject K.W. showed the lowest excretion of vitamin C in the urine. During period 2, especially, it was so much lower than that of the other subjects that it seemed possible there might have been some loss of the vitamin due to poor absorption of the tablets from the intestine. Since his blood figures tended to run high during the whole experiment, it may be that he had a higher kidney threshold for the vitamin than the other three subjects, and that during period 2 this factor was also operating to cause a low excretion.

Comparison of periods 2 and 4. As indicated in table 4, three of the subjects showed a lower excretion of the vitamin during period 4 (cabbage) than during period 2 (vitamin C tablets), the exception being subject K.W. The blood figures for two of the subjects were higher during period 4, and for the other two they were the same. The low blood figure for subject D.P. in period 2 cannot be explained. These results would seem to indicate that the vitamin C of the cabbage was utilized as well, or possibly better, than that in the tablets.

As previously stated, the substitution of cabbage in the basal diet in period 4 changed the calculated acid-base balance of the diet from practically neutral to slightly acid. This substitution, however, did not result in a significant change in the pH of the urine (table 4). The lower excretion of vitamin C during period 4 than during period 2 could be explained as being the result of either decreased absorption or increased retention. It cannot, however, be explained on the basis of increased retention as related to increased base in the diet.

Comparison of periods 2 and 6. Table 4 shows that three of the subjects excreted less vitamin C during period 6 (tomato) than during period 2 (vitamin C tablets), the exception again being subject K.W. The blood figures for three of the subjects were the same in both periods, and for the fourth subject (D.P.) the figure was higher in period 6. It therefore appears that the vitamin C of the tomato juice was also utilized as well, or possibly better, than that in the tablets.

As shown in figure 1, the excretion of vitamin C in the urine of three of the subjects had apparently not quite reached a base line at the end of period 6. These same three subjects showed a lower excretion of the vitamin at the end of period 6 than at the end of period 2. The exception was subject I.H. whose blood figure was slightly lower than those of the other three subjects. Since the blood vitamin C levels of all three subjects were maintained as well in period 6 as in period 2 the decreased excretion in period 6 could again (as for period 4) be explained on the basis of decreased absorption or increased retention. It does not seem probable, however, that there was any increased retention due to the presence of the small amount of extra base which was added to the diet when tomato juice was substituted for apricot juice. On the last day of period 6 this extra base amounted to only 6 cc. of normal solution and produced no change in the pH of the urine. Also no effect on the excretion of vitamin C was noted as the result of day-to-day variations in the amount of tomato juice fed.

Comparison of periods 4 and 6. Judging from the results given in table 4, two of the subjects showed a slightly lower excretion of vitamin C in period 6 (tomato) than in period 4 (cabbage). For the other two the excretion was slightly higher. For three of the subjects the blood figures were practically the same in the two periods, the exception being subject R.B., whose blood figure was higher on cabbage. These results would seem to indicate that the utilization of the vitamin C of cabbage and tomato juice was about equal in these four subjects.

Raw cabbage and tomato juice as sources of vitamin C in the diet. The above results show that, for healthy persons, raw cabbage can be a good source of vitamin C in the diet. However, the conditions under which it is eaten in the average home are quite different from those existing in this experiment. The wide range of vitamin C values secured in the analyses of different heads of cabbage (23.11 to 55.83 mg. per 100 gm.) made in the present study shows that this vegetable is an extremely variable source of the vitamin when it has been stored in a household refrigerator for more than a few days. According to E. F. Murphy,[7] the vitamin C of cabbage is well preserved when the vegetable is stored at 0°C., but when a housewife buys a head of cabbage at the grocery she has no knowledge of the conditions under which it has been stored or how long it has been out of storage. A previous study by one of us (Clayton, '40) showed that cabbage also loses its vitamin C quite rapidly when shredded and mixed with mayonnaise.

Table 2 shows that there was also a considerable range in the vitamin C values secured for different jars of the tomato juice used in this experiment. However, the range was not nearly as great as was shown in the cabbage analyses. Moreover, tomato juice is ordinarily consumed soon after a jar is opened and it is not usually subjected to further treatment which may cause a loss of the vitamin.

[7] Personal communication.

SUMMARY

The availability of the vitamin C in raw cabbage and home-canned tomato juice was studied on four young, healthy subjects. The utilization of the vitamin C of each test food was compared with that of pure vitamin C tablets. The basal diet used was neutral in reaction and the subjects were saturated before each test period. Judging from the results of both blood and urine analyses, the vitamin C of both raw cabbage and tomato juice was utilized as well as, or possibly better than, that in the tablets. An average of 116 gm. of the cabbage or 208 ml. (7/8 cup) of the tomato juice used in this experiment provided 50 mg. of vitamin C.

LITERATURE CITED

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydro-ascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., vol. 126, p. 771.

CLAYTON, MARY M. 1940 The relation of the dietary habits and food resources of Maine people to their state of nutrition with respect to vitamin C. Maine Agr. Exp. Sta. Bul. 400, p. 230.

CLAYTON, MARY M., AND MARIE T. FOLSOM 1940 A method for the study of the availability for human nutrition of the vitamin C in foods, with an application to the study of the potato. J. Home Econ., vol. 32, p. 390.

GODDARD, VERZ R., AND RUTH A. PRESTON 1938 Effect of diet on vitamin C output. J. Home Econ., vol. 30, p. 482.

HAWLEY, ESTELLE E., D. J. STEPHENS AND G. ANDERSON 1936 The excretion of vitamin C in normal individuals following a comparable quantitative administration in the form of orange juice, cevitamic acid by mouth and cevitamic acid intravenously. J. Nutrition, vol. 11, p. 135.

INGALLS, T. H. 1937 Studies on the urinary excretion and blood concentration of ascorbic acid in infantile scurvy. J. Pediatrics, vol. 10, p. 577.

MURPHY, ELIZABETH F. 1942 The ascorbic acid content of different varieties of Maine-grown tomatoes and cabbages as influenced by locality, season, and stage of maturity. J. Agr. Res., vol. 64, p. 483.

TODHUNTER, E. NEIGE, AND ALVA S. FATZER 1940 A comparison of the utilization by college women of equivalent amounts of ascorbic acid (vitamin C) in red raspberries and in crystalline form. J. Nutrition, vol. 19, p. 121.

OBSERVATIONS ON INDUCED RIBOFLAVIN DEFICIENCY AND THE RIBOFLAVIN REQUIREMENT OF MAN

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For this study of isolated riboflavin deficiency subjects were maintained on a basal diet containing carbohydrate, protein and fat in conventional proportions and providing approximately 0.35 mg. of riboflavin per 1000 calories. This allowance was sufficient to permit arrangement of an appetizing diet on which subjects could be maintained for several months. It represented also a degree of restriction which may be encountered in the diets of certain population groups of the United States. To construct such a diet it is necessary to exclude milk, cheese, eggs, organ meats, legumes and green leafy vegetables.

METHODS OF STUDY

Selection of subjects. The several studies herewith reported in full or in part were made in the nutrition division of the Rochester State Hospital on subjects who were provided with an ample diet and daily supplements of 2.0 mg. of thiamine, 2.0 mg. of riboflavin, 2.0 mg. of pyridoxine, 5.0 mg. of calcium pantothenate and 40.0 mg. of nicotinamide for several weeks or months. Thus each subject could be presumed to have optimal stores of riboflavin at the beginning of the period of restriction of riboflavin. During the long period of preliminary observation the subjects were examined and were

familiarized with routines and procedures. After this period thirteen subjects were selected for the several studies reported herein. All subjects were willing and able to cooperate. They were divided into four groups: group 1 consisted of four subjects (subjects 1, 2, 3 and 4) who were selected for the study of isolated restriction of riboflavin. In group 2 were two subjects (subjects 5 and 6) who were selected for the study of simultaneous deprivation of thiamine, pyridoxine, pantothenic acid and niacin after development of riboflavin deficiency. In group 3 were two subjects (subjects 7 and 8) who were selected as controls for groups 1 and 2. These subjects received supplements of thiamine, pyridoxine, pantothenic acid and niacin, and in addition, supplements of riboflavin. By varying the amount of the riboflavin supplement it was possible to determine the approximate amount of riboflavin which would be required for maintenance of satisfactory tissue stores of this vitamin. Group 4 consisted of five subjects (subjects 9 to 13) who were maintained on an intake of 0.5 mg. of riboflavin per 1000 calories in connection with another study (Williams et al.). The only source of any factors of the vitamin B complex for the patients of group 4 was the diet.

All subjects had been engaged in activities such as housekeeping, laundering and sewing. During the period of restriction of riboflavin the same types of activity up to reasonable limits of ability were encouraged.

Tests employed. In addition to physical and neurologic examinations, the following determinations were made during the period of preliminary observation and again at intervals during the period of restriction of riboflavin: protein, calcium and phosphorus in the serum, blood lipids both in the postabsorptive state and after a meal of fats (250 gm. of 33% cream and 35 gm. of egg yolk), blood counts, pyruvic acid (Bueding and Wortis, '40), lactic acid (Barker and Summerson, '41), dextrose in the blood after its administration orally or intravenously, and basal metabolic rates. Electrocardiograms were taken approximately every month; estimates of gastrointestinal motility were made by taking roentgenograms

½, 1, 2, 3 and 5 hours after administration of a meal of barium sulfate. For the test of gastrointestinal motility the subject was maintained in the postabsorptive state and in the sitting or standing position during the period of 5 hours. The eyes of subjects of group 1 were examined every month with the slit lamp microscope; the subjects of groups 2 and 3 were examined with the slit lamp microscope at the end of the period of study.

The urine was analyzed for content of riboflavin every week. Approximately once each month a test dose of 2.0 mg. of sodium riboflavin was administered subcutaneously and excretion of riboflavin in the ensuing 4 hours was determined. Determination of excretion of the test dose of riboflavin was performed with the subject in the postabsorptive state. The fluorometric method of Ferrebee ('40) was used for determinations of riboflavin in the urine.

The test dose of 2.0 mg. of sodium riboflavin was selected on the assumption that the requirement for this vitamin was approximately 2.0 mg. per day for the adult, moderately active woman. In the light of data herewith presented it would appear that a dose of 1.0 mg. of sodium riboflavin administered subcutaneously would be a more satisfactory test dose for the adult whose minimal requirement would probably be between 1.5 and 3.0 mg. of riboflavin. Nevertheless, for the purpose of uniformity in collection of data we have continued to use a dose of 2.0 mg. in all tests.

DIET AND DIETARY SUPPLEMENTS

The standard diet provided for subjects of groups 1, 2 and 3 was composed of foods which commonly appear on American tables. Its composition is given in tables 1 and 2. The diet of subjects of group 4 differed from that of subjects of groups 1, 2 and 3 only in the bread component. The bread used for subjects 9 and 10 of group 4 was made of a whole wheat flour; that for subjects 11, 12 and 13 of group 4 was composed of a white flour to which had been added thiamine (2.0 mg. per pound), nicotinic acid (15 mg. per pound) and nonfat milk

solids (6 pounds per 100 pounds of flour). All diets were analyzed for content of riboflavin by a fluorometric method which was essentially that of Conner and Straub ('41), the values obtained checking closely with microbiologic assays.

TABLE 1

Composition of three variations of the standard diet.[1]

FOOD	STATE OF PREPARATION	AMOUNT OF DIET		
		1	2	3
		gm.	*gm.*	*gm.*
Bread	Special recipe	230	230	230
Butter	As purchased	45	45	45
Cream, 35%	As purchased	75	75	75
Beef, lean	Roasted	100	100	75
Sucrose	As purchased	50	50	50
Cake	Special recipe	50	50	50
Potato, Irish	Baked	75	75	100
Rice, polished, dry	Boiled	25	25	25
Flour	Patent, white	10	10	10
Jelly	As purchased	20	20	20
Puffed rice	Commercial (unfortified)	15	...	...
Carrots	Canned	75	...	...
Lettuce	Fresh	30	...	...
Pineapple	Canned	80	80	...
Gelatin, 20% solution	Special recipe	100	...	100
Tomato	Canned	75	...	...
Apricots, dried, sulfured	Cooked	30	...	...
Farina, dry	Cooked	...	15	15
Applesauce	Cooked	...	100	100
Lemon juice	Fresh	...	20	20
Celery	Fresh	...	30	30
Corn	Canned	...	50	...
Cottage cheese	Washed, skimmed	...	35	35
Beets	Canned	...	...	50

[1] Each diet was repeated every third day.

Caloric intake was adjusted to the requirement of each subject by giving fractions or multiples of the standard diet. By this means the ratio of riboflavin to calories was maintained nearly constant. Approximately 58% of the calories were derived from carbohydrate, 12% from protein and 30% from fat.

Diet of subjects of group 1 (isolated restriction of riboflavin). The standard diet was supplemented with a fortified halibut liver oil, 0.2 gm. (providing 10,000 U.S.P. units of vitamin A and 4000 U.S.P. units of vitamin D); ascorbic acid, 80 mg.; thiamine hydrochloride, 2.5 mg.; pyridoxine, 2.0 mg.; calcium pantothenate, 5.0 mg.; nicotinamide, 20 mg.; inositol,

TABLE 2

Approximate composition of standard diets.

COMPOSITION OF DIET	DIET			
	1	2	3	Composite
Wet weight, gm.	1085	1190	1030	1135
Dry weight, gm.[1]	512	487	497	496
Carbohydrate, gm.[2]	336	323	323	327
Protein, gm.	67	70	65	67
Fat, gm.	76	76	73	75
Calories	2296	2256	2227	2260
Thiamine, mg.[3]	0.55	0.48	0.43	0.48
Ratio of thiamine to calories (mg. per 1000 calories)	0.24	0.21	0.20	0.21
Riboflavin, mg.[4]	0.85	0.84	0.76	0.76
Ratio of riboflavin to calories (mg. per 1000 calories)	0.37	0.37	0.34	0.34
Pantothenic acid, mg.[5]	1.93	2.20	1.72	2.17
Ratio of pantothenic acid to calories (mg. per 1000 calories)	0.84	0.99	0.77	0.96

[1] Air dried at 60°C.

[2] Chatfield, Charlotte and Adams, Georgian: Proximate composition of American food materials. U. S. D. A. No. 549, June, 1940.

[3] Fluorometric assay (thiochrome method of Hennessy).

[4] Fluorometric assay (method of Conner and Straub).

[5] Microbiologic assay. (We are indebted to Dr. Helen T. Parsons, Professor of Home Economics, University of Wisconsin, for analysis of the dried diet for pantothenic acid.)

0.2 gm.; choline chloride, 0.25 gm.; ferrous sulfate, 0.2 gm.; and tricalcium phosphate, 0.6 gm. So far as it could be judged the diet of this group of subjects was adequate in nutrients other than riboflavin.

Diet of subjects of group 2 (restriction of riboflavin and other members of the vitamin B complex). The standard diet

was supplemented with a fortified halibut liver oil, 0.2 gm. (providing 10,000 U.S.P. units of vitamin A and 4000 U.S.P. units of vitamin D); ascorbic acid, 80 mg.; ferrous sulfate, 0.2 gm.; and tricalcium phosphate, 0.6 gm. The diet was deficient, so far as it could be judged, in vitamins of the B complex; it was known to be deficient in thiamine and riboflavin.

Diet of subjects of group 3 (control subjects for subjects of groups 1 and 2). The standard diet was supplemented with thiamine, 2.5 mg.; riboflavin, 1.0 to 3.0 mg.; pyridoxine, 2.0 mg.; calcium pantothenate, 5.0 mg.; nicotinamide, 20.0 mg.; inositol, 0.2 gm.; choline chloride, 0.25 gm.; ferrous sulfate, 0.2 gm.; tricalcium phosphate, 0.6 gm.; ascorbic acid, 80 mg.; and a fortified halibut liver oil, 0.2 gm. (providing 10,000 U.S.P. units of vitamin A and 4000 U.S.P. units of vitamin D). The diet of these subjects was adequate, so far as it could be judged, in every respect.

Diet of subjects of group 4 (less severe restriction of riboflavin). The diet was supplemented with a fortified halibut liver oil, 0.2 gm. (providing 10,000 U.S.P. units of vitamin A and 4000 U.S.P. units of vitamin D); ascorbic acid, 80 mg.; ferrous sulfate, 0.2 gm.; and tricalcium phosphate, 0.6 gm. The diet of these subjects was known to be slightly restricted in content of both thiamine (0.45 mg. per 1000 calories) and riboflavin (0.5 mg. per 1000 calories).

OBSERVATIONS

Isolated deficiency of riboflavin (group 1). The period of restriction of riboflavin began August 18, 1941, and extended to June 2, 1942 (288 days). The subjects (subjects 1, 2, 3 and 4) had only occasional transient complaints and voiced no objection to the regimen. They had good appetites and felt well to the end of the period. The physical and neurologic examinations gave consistently negative results. The concentrations of pyruvic acid, lactic acid and dextrose in the blood were never abnormally elevated after administration of dextrose orally or intravenously. It was noted that basal

metabolic rates remained normal; anemia did not develop; electrocardiograms were unchanged; gastrointestinal motility was not impaired; values for calcium, phosphorus and protein in the blood remained within normal limits; values for lipids in the blood plasma both in the postabsorptive state and after a meal of fat were not abnormal.

Vascularization of the cornea did not develop. An occasional superficial punctate ulceration of the cornea was observed but this was invariably healed on subsequent examinations. They apparently were of the incidental type frequently seen among normal subjects. No complaints were voiced referable to the eyes, although the frequent examinations of the eyes might have caused concern in that regard.

Ulceration, maceration or excrescences at the corners of the mouth, at the nasolabial folds, beneath the pinna of the ears or of any of the cutaneous folds were not observed. The tongue, the gums and the vermilion border of the lips remained essentially normal.

Data on the intake of food, intake and excretion of riboflavin and excretions after a test dose of riboflavin are presented in tables 3 and 4.

Deficiency of riboflavin and other members of the B complex (group 2). The period of restriction of vitamins of the B complex in this experiment began September 12, 1941, and extended through May 15, 1942 (246 days). With these subjects (5 and 6) signs, symptoms and metabolic defects of thiamine deficiency developed within 100 days. The deficiency syndrome was not essentially different from the syndrome of chronic moderate restriction of thiamine which we have described in previous papers (Williams et al., '42). That the signs, symptoms and metabolic defects observed were caused by deficiency of thiamine and not by deficiency of riboflavin is indicated by the fact that in the period of May 16 to June 1, 1942, administration of 15 mg. of thiamine hydrochloride orally per day was associated with disappearance of signs, symptoms and metabolic defects which previously had developed. Physical evidence of deficiency of riboflavin was not observed.

TABLE 3

Isolated restriction of riboflavin: Data on intake and excretion of riboflavin (group 1).

DURATION OF PERIOD	CARBOHYDRATES	PROTEIN	FAT	CALORIES	RIBOFLAVIN	SUPPLEMENT OF RIBOFLAVIN	EXCRETION OF RIBOFLAVIN	
							Ordinary, in 24 hr.	In 4 hr. after test dose [2]
days [1]	*gm.*	*gm.*	*gm.*		*µg.*	*µg.*	*µg.*	*µg.*
Subject 1: Woman, aged 42 years, 158 cm. tall, weighing 62.5 kg.								
End of period of preliminary observation								
10	350	75	70	2330	720	3000	2375	...
23	340	76	73	2321	740	1000	318	...
39	370	74	82	2514	780	....	118	485
Period of restriction of riboflavin								
33	340	69	70	2226	685	....	95	305
61	325	64	68	2168	685	....	110	252
87	331	67	67	2195	658	....	133	133
123	333	66	72	2244	689	....	94	134
156	336	66	75	2283	698	....	90	116
200	329	66	78	2282	699	....	79	153
233	344	67	79	2355	699	....	100	231
261	342	65	77	2321	655	....	95	103
288	361	66	77	2401	676	....	73	121
Subject 2: Woman, aged 25 years, 155 cm. tall, weighing 70.4 kg.								
End of period of preliminary observation								
10	368	75	93	2609	780	3000	2770	...
23	370	70	85	2525	790	1000	686	...
39	365	65	78	2422	740	....	149	485
Period of restriction of riboflavin								
33	338	69	70	2258	736	....	106	305
61	332	65	69	2209	687	....	118	178
87	330	67	67	2191	685	....	94	210
123	259	50	53	1713	546	....	99	119
156	251	50	56	1708	563	....	73	91
200	256	50	58	1746	554	....	67	115
233	267	50	59	1799	544	....	106	86
261	259	50	59	1767	529	....	117	47
288	261	50	58	1766	533	....	61	72

[1] All tests were usually performed within 3 days of the period indicated.

[2] 2.0 mg. of sodium riboflavin subcutaneously; excretion for 4 hours.

TABLE 4

Isolated restriction of riboflavin: Data on intake and excretion of riboflavin (group 1).

DURATION OF PERIOD	CARBOHYDRATES	PROTEIN	FAT	CALORIES	RIBOFLAVIN	SUPPLEMENT OF RIBOFLAVIN	EXCRETION OF RIBOFLAVIN	
							Ordinary, in 24 hr.	In 4 hr. after test dose [2]
days [1]	*gm.*	*gm.*	*gm.*		*µg.*	*µg.*	*µg.*	*µg.*
Subject 3: Woman, aged 32 years, 148 cm. tall, weighing 63.2 kg.								
End of period of preliminary observation								
10	340	70	90	2450	750	3000	2330	....
23	370	82	85	2573	760	1000	375	....
39	325	95	93	2513	784	....	99	419
Period of restriction of riboflavin								
33	317	68	70	2170	715	....	141	348
61	313	65	69	2133	692	....	147	266
87	320	67	67	2151	665	....	124	184
123	313	66	72	2164	689	....	102	167
156	245	50	57	1693	571	....	93	195
200	240	50	58	1682	554	....	81	167
233	254	50	59	1747	540	....	108	180
261	304	64	74	2138	647	....	103	148
288	325	66	76	2248	658	....	74	277
Subject 4: Woman, aged 41 years, 158 cm. tall, weighing 45.5 kg.								
End of period of preliminary observation								
7	360	73	84	2488	761	2000	1193	....
8	350	70	80	2400	750	....	193	1315
Period of restriction of riboflavin								
23	348	71	91	2495	771	....	138	330
51	470	96	128	3146	1000	....	98	295
77	496	100	132	3572	1008	....	83	184
113	535	100	109	3521	941	....	62	287
146	518	99	111	3467	982	....	66	140
190	353	66	77	2369	680	....	65	230
223	355	67	79	2399	684	....	93	385
261	345	66	77	2337	685	....	80	261
288	353	66	77	2369	661	....	63	306

[1] All tests were usually performed within 3 days of day indicated.

[2] 2.0 mg. of sodium riboflavin subcutaneously; excretion for 4 hours.

TABLE 5

Restriction of vitamins of the B complex: Data on intake and excretion of thiamine and riboflavin (group 2).

DURATION OF PERIOD	DIET						EXCRETION OF THIAMINE		EXCRETION OF RIBOFLAVIN		BIOCHEMICAL STUDIES,[3] MG. PER 100 CC. OF BLOOD		
	Carbohydrate	Protein	Fat	Calories	Thiamine	Riboflavin	Ordinary, in 24 hr.	In 4 hr. after test dose [2]	Ordinary, in 24 hr.	In 4 hr. after test dose [2]	Pyruvic acid	Lactic acid	Dextrose
days [1]	*gm.*	*gm.*	*gm.*		*µg.*	*µg.*	*µg.*	*µg.*	*µg.*	*µg.*			
Subject 5: Woman, aged 30 years, 152 cm. tall, weighing 46.4 kg. End of period of preliminary observation													
10	238	60	100	2092	819	...	99	376	201	1020	1.2	12.0	149
Period of restricted constant intake													
27	310	70	80	2240	483	703	14	197	84	424	...	....	...
60	304	68	87	2271	516	725	8	147	84	286	...	....	...
95	312	68	73	2177	489	674	8	163	85	282	...	....	...
130	227	50	56	1612	334	569	8	...	77	....	1.9	14.8	174
151	226	51	55	1603	334	558	5	169	65	252	...	....	...
194	236	50	60	1684	352	570	4	115	69	379	2.6	18.6	128
246	231	50	58	1646	367	525	8	127	76	269	1.9	14.2	132
Period of treatment with thiamine hydrochloride [4]													
15	242	50	59	1699	362	530	...	514	...	220	1.6	12.7	105
Subject 6: Woman, aged 44 years, 157 cm. tall, weighing 67.0 kg. End of period of preliminary observation													
10	240	63	105	2157	819	...	120	230	385	740	1.2	12.6	144
Period of restricted constant intake													
27	300	70	70	2110	518	744	32	109	151	357	...	....	...
60	302	66	66	2066	501	684	9	94	99	245	...	....	...
95	231	51	53	1605	367	530	9	63	89	170	...	....	...
130	236	50	57	1657	334	569	10	...	84	....	2.0	14.8	134
157	231	51	55	1623	344	558	5	102	74	185	...	....	...
194	238	51	60	1696	364	575	7	80	94	182	2.1	13.0	145
246	236	50	58	1666	356	520	9	98	95	125	1.9	19.6	144
Period of treatment with thiamine hydrochloride [4]													
15	216	47	56	1556	334	511	...	692	...	149	1.4	12.4	124

[1] All tests were usually performed within 3 days of day indicated.
[2] 60 minutes after administration of dextrose orally.
[3] Test dose: 1.0 mg. of thiamine hydrochloride plus 2.0 mg. of sodium riboflavin subcutaneously.
[4] 15.0 mg. of thiamine hydrochloride orally per day.

Data on the intake of food, intake and excretion of riboflavin and excretion after a test dose of riboflavin are contained in table 5.

Riboflavin requirement of man (group 3, control for groups 1 and 2). The subjects (subjects 7 and 8) were maintained on the standard diet from August 18, 1941, to June 2, 1942 (288 days). Since these subjects were controls for subjects of groups 1 and 2, it seemed important to supply crystalline riboflavin in amounts clearly above the minimal requirement and, therefore, for the first few months 2.0 or 3.0 mg. of crystalline riboflavin were provided daily. Later, in view of the negative results obtained in the study of group 1, it appeared practical to reduce the intake of riboflavin of the subjects of group 3 to a level which would provide some data on the riboflavin intake capable of just preventing depletion of tissue stores of this vitamin. Therefore, the supplement of riboflavin was reduced to 1.0 mg. per day. Throughout the period of study no evidence of deficiency disease was observed in these subjects.

Data on the intake and excretion of riboflavin, excretions after test doses of riboflavin and intake of food are contained in table 6.

The data on the urinary excretion of riboflavin after administration of test doses of this vitamin to the subjects of group 3 seem to indicate that when the intake of riboflavin was 0.8 mg. per 1000 calories, there was no evidence of depletion of tissue stores. Therefore, we are prompted to present in part data of a study, not yet reported, in which the intake level of riboflavin of five subjects was maintained at approximately 0.5 mg. per 1000 calories.

Basal diet containing 0.5 mg. of riboflavin per 1000 calories (group 4). The diet of these five subjects (subjects 9 to 13) contained 0.45 mg. of thiamine and 0.5 mg. of riboflavin per 1000 calories. It was fed from September 12, 1941, to June 2, 1942 (264 days). Evidence of excretions and biochemical

• 15.0 mg. of thiamine hydrochloride orally per day.

TABLE 6

Riboflavin requirement of man: Data on control subjects (group 3) for study of induced riboflavin deficiency.

DURATION OF PERIOD	DIET						EXCRETION OF RIBOFLAVIN	
	CARBOHYDRATE	PROTEIN	FAT	CALORIES	RIBOFLAVIN	SUPPLEMENT OF RIBOFLAVIN	Ordinary, in 24 hr.	In 4 hr. after test dose [2]
days [1]	*gm.*	*gm.*	*gm.*	*gm.*	*µg.*	*µg.*	*µg.*	*µg.*
Subject 7: Woman, aged 24 years, 172 cm. tall, weighing 66.8 kg.								
78	327	67	67	2179	675	1000	480	....
167	331	66	72	2236	685	2000	1140	....
196	329	68	77	2281	700	1000	494	719
220	342	68	80	2360	702	2000	1250	634
242	329	66	78	2282	678	3000	1810	1155
245	332	66	77	2285	668	1000	580	....
259	321	66	77	2241	651	1000	458	....
264	328	66	77	2269	664	1000	518	541
288	329	68	74	2254	670	1000	572	526
Subject 8: Woman, aged 46 years, 163 cm. tall, weighing 63.2 kg.								
78	296	66	67	2051	665	1000	463	....
167	277	62	69	1977	642	2000	1240	....
196	300	65	77	2153	687	1000	540	432
220	314	68	80	2248	701	2000	1095	490
242	305	65	78	2182	673	3000	1680	1000
245	293	61	73	2073	651	1000	715	....
259	304	66	77	2173	650	1000	555	....
264	312	66	77	2205	665	1000	465	311
288	310	65	79	2211	648	1000	544	586

[1] All tests were usually performed within 3 days of day indicated.

[2] 2.0 mg. of sodium riboflavin subcutaneously; excretion for 4 hours.

studies indicated that for these subjects this intake of thiamine was not satisfactory. However, clear-cut symptoms of any deficiency disease were not observed.

Data on intake of food, intake and excretion of riboflavin and excretions after test doses of riboflavin are contained in tables 7 and 8.

COMMENT

The technical procedures of this study of induced riboflavin deficiency differ in at least three essential respects from that of Sebrell and Butler ('39). In the first instance, our subjects

were given large amounts of riboflavin for a considerable period prior to the period of restriction of riboflavin and, therefore, presumably they began the period of restriction with optimal stores of riboflavin in the tissues. The subjects of Sebrell and Butler had not received extra riboflavin prior to the period of restriction. Indeed it is not unlikely that their

TABLE 7

Riboflavin requirement of man: Excretion of riboflavin on intake of approximately 0.5 mg. per 1000 calories (group 4).

DURATION OF PERIOD	DIET					EXCRETION OF RIBOFLAVIN	
	Carbohydrate	Protein	Fat	Calories	Riboflavin	Ordinary, in 24 hr.	In 4 hr. after test dose [2]
day [1]	*gm.*	*gm.*	*gm.*		*µg.*	*µg.*	*µg.*
Subject 9: Woman, aged 34 years, 158 cm. tall, weighing 51.9 kg.							
End of period of preliminary observation							
10	327	68	83	2327	....	296	485
Period of restriction of riboflavin							
30	307	66	87	2275	1124	115	355
63	328	69	88	2380	1056	117	243
98	341	68	74	2302	1025	48	236
132	273	58	63	1891	936	93	...
160	304	71	70	2130	1095	100	241
199	320	80	80	2320	1078	107	450
243	324	66	80	2280	1023	110	214
263	337	66	79	2323	1030	...	195
Subject 10: Woman, aged 21 years, 157.5 cm. tall, weighing 60 kg.							
End of period of preliminary observation							
10	276	63	105	2301	....	529	548
Period of restriction of riboflavin							
30	328	65	89	2373	1084	212	340
63	344	69	87	2435	1054	173	262
98	344	69	75	2327	1009	133	304
132	420	84	96	2880	1363	144	...
160	422	85	95	2883	1317	138	230
198	440	84	102	3014	1324	156	303
243	335	68	80	2332	1039	141	205
263	351	67	80	2392	1050	...	244

[1] All tests were usually performed within 3 days of day indicated.

[2] 2.0 mg. of sodium riboflavin subcutaneously; excretion for 4 hours.

TABLE 8

Riboflavin requirement of man: Excretion of riboflavin on intake of approximately 0.5 mg. per 1000 calories (group 4).

DURATION OF PERIOD	DIET					EXCRETION OF RIBOFLAVIN	
	Carbohydrate	Protein	Fat	Calories	Riboflavin	Ordinary, in 24 hr.	In 4 hr. after test dose [2]
day [1]	*gm.*	*gm.*	*gm.*		*µg.*	*µg.*	*µg.*
Subject 11: Woman, aged 37 years, 157 cm. tall, weighing 71.7 kg.							
End of period of preliminary observation							
10	250	60	100	2140	....	369	695
Period of restriction of riboflavin							
30	350	70	66	2274	1045	209	484
63	337	67	67	2219	1018	136	278
98	354	67	72	2332	1037	116	160
132	353	65	75	2347	1073	118	...
160	349	65	75	2331	1074	124	167
199	359	68	79	2419	1069	133	281
243	347	68	79	2371	1050	133	132
263	337	66	77	2305	1043	...	281
Subject 12: Woman, aged 42 years, 173 cm. tall, weighing 64.6 kg.							
End of period of preliminary observation							
10	309	69	83	2259	....	256	665
Period of restriction of riboflavin							
30	330	70	77	2293	1058	123	338
63	292	60	79	2119	1037	85	272
98	314	63	68	2120	968	94	166
132	323	68	75	2339	1082	93	...
160	333	66	74	2262	1060	94	108
199	340	68	80	2352	1092	108	111
243	315	68	78	2234	1035	118	201
263	328	66	77	2269	1045	...	249
Subject 13: Woman, aged 27 years, 159 cm. tall, weighing 70.4 kg.							
End of period of preliminary observation							
10	306	65	84	2240	....	196	572
Period of restriction of riboflavin							
30	345	66	76	2328	1015	87	458
63	343	67	67	2243	1020	99	151
98	324	68	73	2225	1029	93	272
132	313	66	75	2191	1083	79	205
160	308	67	74	2166	1069	106	145
199	322	66	80	2272	1088	120	300
243	300	67	77	2161	1043	101	216
263	325	66	78	2266	1050	...	299

[1] All tests were usually performed within 3 days of day indicated.

[2] 2.0 mg. of sodium riboflavin subcutaneously; excretion for 4 hours.

intakes of riboflavin had been low before the study was undertaken. The presence or absence of tissue stores of riboflavin undoubtedly would have a bearing on the development of clinical signs. In the second instance, restriction of riboflavin in our study was only to 0.7 mg. per day (0.35 mg. per 1000 calories) whereas in the study of Sebrell and Butler the intake of riboflavin was restricted to 0.5 mg. per day. That a slight difference in intake level can result in different effects is illustrated by other observations. Subjects of our studies who received 0.5 mg. of riboflavin per 1000 calories (group 4) apparently had better stores of riboflavin at the end of the period of restriction than did subjects who received 0.35 mg. per 1000 calories (groups 1 and 2). Also, studies of induced thiamine deficiency have shown that slight differences of intake of thiamine may modify greatly the time of onset and the manifestations of thiamine deficiency disease. Finally the subjects of our study of riboflavin deficiency received a test dose of 2.0 mg. of sodium riboflavin approximately once each month and this periodic administration may have inhibited development of abnormal signs.

When the intake of riboflavin of the subjects of group 3 (subjects 7 and 8, table 6) was 3.6 mg. per day 1600 to 1800 μg. were excreted; when the intake was 1.6 mg. per day 400 to 700 μg. were excreted. Other data show that when the intake is 1.0 mg. or less the excretion usually is between 150 and 350 μg. per day.

The data on excretion of riboflavin of subjects of group 3 after administration of test doses of this vitamin seem to indicate that an intake of approximately 1.6 mg. per day (0.8 mg. per 1000 calories from the two-hundred forty-third to the two-hundred eighty-eighth day of this experiment, a period of 45 days) was not associated with depletion of tissue stores of riboflavin.

The data on excretion of test doses of riboflavin of subjects of group 4 (subjects 9 and 10, table 7; subjects 11, 12 and 13, table 8) indicate that on an intake of 1.0 mg. per day

(0.5 mg. per 1000 calories) the tissue stores of riboflavin were depleted only to a slight degree.

The data on excretion of test doses of riboflavin of subjects of groups 1 and 2 (subjects 1 through 6, tables 3, 4 and 5) indicate that on an intake of 0.7 mg. per day (0.35 mg. per 1000 calories) there was unmistakable depletion of tissue stores of riboflavin.

The data thus appear to justify a tentative conclusion that the minimal requirement for riboflavin of these subjects was in the neighborhood of 0.5 mg. per 1000 calories and that it certainly did not exceed 0.8 mg. per 1000 calories. The allowance of riboflavin of 2.2 mg. per day (0.88 mg. per 1000 calories) which was recommended for a moderately active woman by the Food and Nutrition Board of the National Research Council thus appears to provide a liberal margin of safety.

SUMMARY

Four subjects were maintained for 288 days on a standard diet containing only 0.35 mg. of riboflavin per 1000 calories but made adequate in other factors of the vitamin B complex by addition of them in crystalline form. The only evidence of deficiency observed was progressive decrease of excretion of a test dose of 2.0 mg. of sodium riboflavin, indicating progressive depletion of tissue stores of riboflavin. However, in view of the gradual depletion of stores it may be assumed that if this degree of restriction had been continued for a longer period of time, clinical evidence of riboflavin deficiency eventually would have appeared.

Two subjects were maintained on the same standard diet for a period of 246 days but these subjects did not receive supplements of crystalline vitamins and therefore the deficiency involved all factors of the B complex. This moderately severe restriction of the B complex did not produce the clinical syndrome of riboflavin deficiency. However, again a gradual depletion of stored riboflavin was indicated by a progressive decrease of excretion of test doses of riboflavin.

Two subjects maintained on the same standard diet for a period of 288 days were given crystalline riboflavin in addition to other vitamins of the B complex. Evidence of deficiency of any kind was not observed.

Variation of the intake of crystalline riboflavin supplement and determination of excretion of test doses of riboflavin provided evidence that an intake of 0.8 mg. of riboflavin per 1000 calories was not associated with depletion of tissue stores of riboflavin, whereas an intake of 0.35 mg. per 1000 calories definitely was so associated. An intake of 0.5 mg. per 1000 calories appeared to be close to the daily requirement necessary for maintenance of satisfactory tissue stores of riboflavin. This value therefore (0.5 mg. per 1000 calories) appears closely to approximate the minimal daily requirement for riboflavin.

LITERATURE CITED

BARKER, S. B., AND W. H. SUMMERSON 1941 The colorimetric determination of lactic acid in biological materials. J. Biol. Chem., vol. 138, pp. 535–554.

BUEDING, ERNEST, AND HERMAN WORTIS 1940 The stabilization and determination of pyruvic acid in the blood. J. Biol. Chem., vol. 133, pp. 585–591.

CONNER, R. T., AND G. J. STRAUB 1941 Combined determination of riboflavin and thiamin in food products. Indust. & Engin. Chem. (Analyt. ed.), vol. 13, pp. 385–388.

FERREBEE, J. W. 1940 The urinary excretion of riboflavin: fluorometric methods for its estimation. J. Clin. Investigation, vol. 19, pp. 251–256.

SEBRELL, W. H., AND R. E. BUTLER 1939 Riboflavin deficiency in man (ariboflavinosis). Pub. Health Rep., vol. 54, pp. 2121–2131.

WILLIAMS, R. D., H. L. MASON AND R. M. WILDER Evaluation of nutritive contribution of enriched white flour. (In press.)

WILLIAMS, R. D., H. L. MASON, B. F. SMITH AND R. M. WILDER 1942 Induced thiamine (vitamin B_1) deficiency and the thiamine requirement of man: further observations. Arch. Int. Med., vol. 69, pp. 721–738.

FAT EXCRETION BY NORMAL CHILDREN [1]

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From the viewpoint of the nutritionist, the products of food digested in the alimentary canal are not in the body until they have been absorbed through the intestinal wall. It is difficult to determine, precisely, the extent of utilization of food nutriments, inasmuch as materials are secreted and excreted into the gastrointestinal tract as well as absorbed from it. Fecal fat may represent unabsorbed food fat, excreted fat, or both, for, regardless of whether fat is ingested, the feces always contain a measurable amount of lipid material which consists mainly of fatty acids and their salts (soaps), sterols and a small amount of neutral fat or triglyceride (Hill and Bloor, '22).

Of the many factors which have been reputed to affect the excretion of fat in the feces, the most significant are the amounts of bile and pancreatic juice from the intestine (Coffey et al., '40 a, b), the mineral intake (Holt et al., '35), and the type of fat ingested (Blatt and Harris, '37; Frontali, '39; Holt et al., '35). Numerous studies have demonstrated that premature infants absorb fat very poorly (Gordon and McNamara, '41; Tidwell et al., '35), and the newborn are less efficient than older infants in this respect (Gordon and McNamara, '41; Holt et al., '35). Degree of development,

[1] Preliminary reports of part of this work were given before the 100th meeting of the American Chemical Society, Detroit, Michigan, September 9–13, 1940, and before the American Society of Biological Chemists at the meeting of the Federation of American Societies for Experimental Biology, Toronto, Canada, April 26–29, 1939.

therefore, appears to be a factor in fat excretion, at least during the first year of life (Gordon and McNamara, '41; Holt et al., '35; Wang et al., '39).

The early observation that the feces of infants ingesting cow's milk contained larger amounts of soaps than those of babies taking human milk focused the attention of investigators on the interrelationship of mineral and fat utilization. Cow's milk contains about four times the mineral content of human milk and proportional differences exist between the mineral constituents. Differences in mineral constituency occur for other species as well and appear to be related to their different rates of skeletal growth (Telfer, '30). In addition to the deviations in mineral composition, there are variations in fat quality which appear even more significant, not only in the utilization and excretion of fat, but also of the minerals. The quality of various fats is dependent on the fatty acid distribution in the various glycerides which compose them and certain fatty acids are better utilized than others (Hilditch, '40). The length of the carbon chain and the degree of saturation of the fatty acids have been demonstrated as most significant factors in their utilization (Holt et al., '35). Human milk fat is better utilized than butterfat (Holt et al., '35). Human milk fat is less saturated than butterfat (Hoobler and Macy, '39) and has been reported to contain more than twice as much triolein (Holt et al., '35; Frontali, '39). Olive oil and soybean oil, which contain more oleic and other unsaturated fatty acids, are better utilized than the fat of human milk (Holt et al., '35). In this connection, the relative utilization of the calcium soap of oleic acid has been shown to be much greater than that of calcium palmitate, which in turn is greater than that of calcium stearate (Boyd et al., '32). This is also true for the sodium salts of these fatty acids (Hilditch, '40).

The subjects of the present investigation of fat excretion were healthy, growing children between the ages of 4 and 12 years (table 1). They were given diets of high nutritive quality while maintained on continuous metabolic regimes for

from 30 to 225 days. The fat contents of 5-day composites of all the food ingested were determined by the Soxhlet extraction procedure and the lipid partitions of the 5-day composites of feces were determined by the method of Tidwell and Holt ('35), slightly modified (Macy, '42). The foods eaten and

TABLE 1

Physical characteristics of subjects.

SUBJECT	AGE	FIVE-DAY PERIODS IN STUDY	RECUMBENT LENGTH	WEIGHT
	years		*cm.*	*kg.*
I [1]	5	39	106.8	17.68
II [1]	5	45	110.9	19.07
III [1]	6	43	117.1	21.12
IV	8	6	124.4	23.50
V	8	13	136.5	27.64
VI	8	6	119.4	22.92
VII	4	43	108.6	17.99
VIII	5	41	116.0	19.16
IX [1]	6	37	118.6	22.12
X	6	45	120.5	22.62
XI	6	45	120.8	21.24
XII	9	11	120.3	24.39
XIII	8	11	132.2	27.08
XIV [1]	9	11	131.2	28.13
XV	9	11	140.9	29.04
IV [2]	8	30	120.4	24.32
V [3]	8	32	138.0	28.14
XVI	10	11	139.7	31.80
XVII	10	11	144.9	33.64
XVIII	12	11	159.1	41.84
XIX	11	11	138.3	35.75

[1] Girls.

[2] Same child as IV.

[3] Same child as V. During 65 consecutive days this subject was given approximately the same calcium intake as children who were much smaller, to determine the minimal requirement.

details of the experimental procedure, preparation of the samples, and analytical techniques have been published (Macy, '42). The main source of fat in the diets, other than that contained in natural foods, was butterfat.

RESULTS

Fat balances. The fat balances for 513 5-day periods are presented in table 2. The fat intakes ranged from 62 to 113 gm. per day and for all levels of intake the average fat utilization ranged between 96 and 98%. The children excreted an average of 2.3 gm. of lipid material daily, varying between 1.3 and 3.8 gm., which represented 10 to 18% of the fecal dry weight, an average of 13%. The data show a definite trend of increased fat excretion in grams per day with higher fat intakes which resulted in approximately constant per cent

TABLE 2

Average daily fat intakes, excretions and retentions.

SUBJECT	FAT INTAKE		FAT IN FECES		FAT RETENTION	
	Mean	S.D.[1]	Mean	S.D.[1]	Mean	S.D.[1]
	gm.		*gm.*		*gm.*	
I [2]	62.4	3.4	1.7	0.4	60.7	3.1
II [2]	67.7	6.4	2.2	0.4	65.5	6.2
III [2]	67.8	6.3	1.8	0.5	66.0	6.1
IV	69.5	0.0	1.3	0.2	68.2	0.6
V	70.3	0.7	1.9	0.3	68.4	1.2
VI	71.0	0.0	1.8	0.4	69.2	0.4
VII	71.2	8.3	1.6	0.4	69.6	8.1
VIII	71.7	8.0	2.3	0.6	69.4	7.8
IX [2]	75.1	13.0	2.0	0.6	73.1	12.6
X	76.8	10.9	2.4	0.7	74.4	10.9
XI	76.8	10.9	1.8	0.5	75.0	10.6
XII	82.7	2.3	3.1	0.3	79.6	2.6
XIII	82.8	2.3	2.6	0.2	80.2	2.6
XIV [3]	82.8	2.3	2.9	0.3	79.9	2.6
XV	82.8	2.3	3.2	1.2	79.6	3.0
IV [3]	101.1	1.6	3.5	0.7	97.6	1.9
V [4]	101.5	1.4	2.8	0.5	98.7	1.7
XVI	112.8	2.2	3.7	0.6	109.1	2.6
XVII	112.8	2.2	3.7	0.5	109.1	2.8
XVIII	112.8	2.2	3.8	0.3	109.0	2.4
XIX	112.8	2.2	3.0	0.4	109.8	2.5
Average	79.4	16.4	2.3	0.8	77.1	15.9

[1] Standard deviation.
[2] Girls.
[3] Same child as IV.
[4] Same child as V.

retentions [2] of fat (average 97.1%) at all levels of intake. There was some tendency for an increased percentage of fat in the dry weight of the stools with higher levels of intake, but it is not striking.

Although formerly fecal fat was thought to be derived from food fat that had not been digested and absorbed, from bacteria and from trauma of the intestinal mucosa, evidence which has accumulated indicates that it is not unabsorbed material but is, largely, the product of secretion or excretion (Sperry and Bloor, '24) into the intestine. General agreement now is that, under normal conditions, fecal lipids are of endogenous origin and can no longer be regarded as unabsorbed fragments of the diet (Krakower, '34; Shapiro et al., '36). With this wider recognition, confusion has arisen as to the best method of studying fat utilization. Some early workers held the view that the percentage fat content of the dry stool was a physiologic constant, approximately one-third of the fecal dry weight (Hutchison, '19), that could be used as the criterion of fat absorption (Fowweather, '26; Harrison and Sheldon, '27). Our results confirm other recent studies and show that not only is the per cent in the feces quite variable (Holt et al., '35), but in normal children, it comprises much less than one-third of the dry weight (Wang et al., '39). In instances of low fat intake, the results of balance studies do not yield per cent retention figures representative of the true utilization, because the endogenous fecal lipids are so high in proportion to the fat intake; however, for this factor to operate, the fat intake must be quite low, less than 10 gm. per day (Holt et al., '35); barring this situation, generally the per cent retention is valid as a measure of fat utilization.

Fecal fat distribution. During eleven balance periods for each of eight children, the feces were dried from the frozen state under vacuum (Teague et al., '42) and the lipid partition of the fecal fat was investigated. The children's diets contained the same basic foods in identical amounts, but four

[2] For lack of a better term, fat retention is used in the conventional way (i.e., fat intake minus fat in feces).

received additional quantities of butter oil and potato to enhance their caloric intake. As a result, four children received a daily intake of 82.8 gm. of fat, and the four older, larger children received 112.8 gm. (table 3). On a unit weight basis, although the high level group received an additional 30 gm. of fat per day, this amount distributed among children who averaged approximately 9 kg. of body weight in excess of the average weight of the low level group (table 1) provided them with an intake per kilogram of body weight only 0.1 gm. greater than that of the low level group. In terms of total caloric intake, the low-fat group received 36% of their total food calories in the form of fat, and the higher intake group, 43%. The fecal fat excretion on the low and high levels of fat intake represented 27 and 34 Calories per day (1.04 and 0.95 Cal. per kilogram) or 26 and 32% of the total fecal Calories (107 and 106 Cal., respectively).

The results of the fecal fat partition (table 3) show that excretion of free fatty acid, neutral fat, and unsaponifiable material was approximately the same at the two levels of fat intake, which suggests that these components of the fecal fat have an endogenous origin. However, in the four children who received an additional 30 gm. of fat per day, the soap excretion was significantly higher. Further evidence pointing to the possible endogenous nature of the free fatty acids, neutral fat and unsaponifiable matter of the feces is shown by the results calculated on a unit weight basis (table 3). The older and larger children with approximately the same intake per kilogram excreted less of the above three fecal lipids in terms of body weight. However, the excretion of soap averaged slightly more on the same basis. From these results it appears that the increased lipid excretion resulting from the larger fat intake consisted mainly of soap. The importance of soaps in the fat excretion of children is shown by the percentage distribution of the various fecal lipids (table 3). Approximately one-half of the fecal lipid is soap. The next largest component is the unsaponifiable fraction which contributes about one-third of the total. Free fatty acids and neutral fat each make up less than 10% of the total.

TABLE 3

Average daily fat intake and fecal fat partitions for eight children during eleven consecutive 5-day periods[1]

	SUBJECTS ON LOW LEVEL INTAKE					SUBJECTS ON HIGH LEVEL INTAKE				
	XII	XIII	XIV[2]	XV	Average	XVI	XVII	XVIII	XIX	Average
Fat intake										
Grams per day	82.8 ±2.3	82.8 ±2.3	82.8 ±2.3	82.8 ±2.3	82.8 ±2.3	112.8 ±2.2	112.8 ±2.2	112.8 ±2.2	112.8 ±2.2	112.8 ±2.2
Gm./kg. body weight	3.4 ±.1	3.0 ±.1	2.9 ±.1	2.8 ±.1	3.1 ±.2	3.6 ±.1	3.4 ±.1	2.7 ±.1	3.2 ±.1	3.2 ±.3
Fecal fat										
Grams per day	3.1 ±0.3	2.6 ±0.2	2.9 ±0.3	3.2 ±1.2	2.9 ±0.7	3.7 ±0.6	3.7 ±0.5	3.8 ±0.3	3.0 ±0.4	3.6 ±0.5
Gm./kg. body weight	0.13 ±0.01	0.09 ±0.03	0.10 ±0.03	0.11 ±0.04	0.11 ±0.03	0.12 ±0.02	0.11 ±0.02	0.09 ±0.02	0.08 ±0.03	0.10 ±0.02
Fecal fat partition										
Free fatty acid										
Grams per day	0.33 ±.08	0.26 ±.03	0.29 ±.04	0.26 ±.03	0.28 ±.07	0.31 ±.03	0.28 ±.06	0.31 ±.07	0.28 ±.06	0.30 ±.02
Mg./kg. body weight	13.7 ±2.4	9.5 ±.3	10.2 ±1.6	8.9 ±1.0	10.6 ±2.3	9.6 ±1.9	8.5 ±.7	7.5 ±.8	8.0 ±.5	8.4 ±1.3
Per cent total fecal fat	10.7 ±1.6	10.1 ±1.1	10.1 ±2.1	8.6 ±1.6	9.9 ±1.7	8.3 ±0.7	7.7 ±1.1	8.2 ±1.1	9.4 ±1.0	8.4 ±1.2
Unsaponifiable fat										
Grams per day	0.93 ±.09	0.86 ±.06	1.09 ±.13	0.99 ±.17	0.97 ±.12	1.16 ±.32	1.13 ±.02	1.13 ±.14	0.80 ±.15	1.06 ±.21
Mg./kg. body weight	38.2 ±1.6	31.7 ±3.0	38.8 ±4.1	34.3 ±4.6	35.7 ±4.9	36.6 ±9.9	33.5 ±2.7	27.1 ±2.0	22.4 ±3.8	29.9 ±7.7
Per cent total fecal fat	29.9 ±1.8	33.6 ±2.7	38.0 ±5.8	32.9 ±6.6	33.6 ±5.4	31.2 ±3.3	30.5 ±3.4	29.8 ±2.2	26.3 ±5.0	29.4 ±4.0
Soap										
Grams per day	1.61 ±.14	1.12 ±.10	1.27 ±.36	1.62 ±.93	1.40 ±.55	1.77 ±.26	2.13 ±.39	2.17 ±.23	1.64 ±.33	1.93 ±.36
Mg./kg. body weight	65.9 ±6.2	41.2 ±5.3	45.6 ±14.8	55.9 ±33.6	52.1 ±20.7	55.5 ±9.3	63.3 ±12.1	51.8 ±5.9	46.1 ±8.2	54.2 ±10.7
Per cent total fecal fat	51.6 ±3.9	43.6 ±4.2	43.1 ±8.4	48.3 ±6.9	46.7 ±6.9	47.8 ±2.7	56.6 ±4.1	56.7 ±3.4	53.4 ±6.5	53.6 ±5.5
Neutral fat										
Grams per day	0.25 ±.07	0.32 ±.08	0.25 ±.13	0.33 ±.18	0.29 ±.12	0.46 ±.11	0.19 ±.06	0.20 ±.04	0.32 ±.12	0.30 ±.12
Mg./kg. body weight	10.1 ±3.4	11.8 ±2.8	8.8 ±4.5	11.5 ±6.3	10.6 ±4.3	14.6 ±2.7	5.7 ±1.5	4.8 ±0.8	9.1 ±2.8	8.6 ±4.3
Per cent total fecal fat	7.8 ±2.2	12.6 ±2.4	8.8 ±4.8	10.1 ±2.7	9.8 ±3.6	12.7 ±2.3	5.2 ±1.1	5.3 ±0.4	10.8 ±3.9	8.5 ±4.1

[1] Values given are means or means plus or minus standard deviations.

[2] Girl.

SUMMARY

The fat excretion of nineteen healthy children, ages 4 to 12 years, was investigated over 513 5-day periods while they were ingesting diets of high nutritive quality. Fat intakes ranged from 62 to 113 gm. per day and the utilization varied from 96 to 98%. An average daily excretion of 2.3 gm. of lipids, which represented an average of 13% of the fecal dry weight, was observed.

Feces dried from frozen state under vacuum were used in determinations of the fecal lipid partition for eight children over eleven consecutive 5-day periods. Four children received 83 gm. (3.1 gm./kg.) of fat per day and the other four, 113 gm. (3.2 gm./kg.). Excretion of free fatty acid, neutral fat and unsaponifiable material was approximately equal for all the subjects but the soap excretion was significantly higher for the children with the larger fat intakes, 1.93 ± 0.36 gm. (54.2 mg./kg.) as compared with 1.40 ± 0.55 gm. (52.1 mg./kg.). For all the children soaps comprised approximately 50% of the total fecal fat, unsaponifiable fat represented approximately 30%, and the remainder was almost equally divided between free fatty acid and neutral fat.

LITERATURE CITED

BLATT, M. L., AND E. H. HARRIS 1937 The substitution of olive oil for butter fat in infant feeding. J. Pediatrics, vol. 11, p. 455.

BOYD, O. F., C. L. CRUM AND J. F. LYMAN 1932 The absorption of calcium soaps and the relation of dietary fat to calcium utilization in the white rat. J. Biol. Chem., vol. 95, p. 29.

COFFEY, R. J., R. C. MANN AND JESSE L. BOLLMAN 1940 a The effect of the exclusion of bile on the absorption of foodstuffs. Am. J. Dig. Dis., vol. 7, p. 143.

———— 1940 b The influence of the pancreas on the utilization of foodstuffs. Am. J. Dig. Dis., vol. 7, p. 144.

FOWWEATHER, F. S. 1926 The determination of the amounts and the composition of fats of feces. II. The composition of the fats of the feces of the normal adult as ascertained by the wet method together with some results in certain pathological conditions. Brit. J. Exp. Path., v. 7, p. 15.

FRONTALI, G. 1939 Oils as substitutes for butter fat in infant feeding. J. Pediatrics, vol. 14, p. 290.

GORDON, H. H., AND HELEN MCNAMARA 1941 Fat excretion of premature infants. I. Effect on fecal fat of decreasing fat intake. Am. J. Dis. Child., vol. 62, p. 328.

HARRISON, G. A., AND W. C. H. SHELDON 1927 Fecal fat analysis in children. Arch. Dis. Childhood, vol. 2, p. 338.

HILDITCH, T. P. 1940 The Chemical Constitution of Natural Fats. John Wiley and Sons, Inc., New York.

HILL, ELSIE, AND W. R. BLOOR 1922 Fat excretion. J. Biol. Chem., vol. 53, p. 171.

HOLT, L. EMMETT, JR., H. C. TIDWELL, C. M. KIRK, DOROTHEA M. CROSS AND SARAH NEALE 1935 Studies in fat metabolism. I. Fat absorption in normal infants. J. Pediatrics, vol. 6, p. 427.

HOOBLER, B. R., AND ICIE G. MACY 1939 Milk. The Cyclopedia of Medicine, Surgery and Specialties, p. 168, F. A. Davis Co., Philadelphia.

HUTCHISON, H. S. 1919 Fat metabolism in health and disease with special reference to infancy and childhood. Quart. J. Med., vol. 13, p. 277.

KRAKOWER, A. 1934 Fecal "fat" and its relation to fat in the diet. Am. J. Physiol., vol. 107, p. 49.

MACY, ICIE G. 1942 Nutrition and Chemical Growth in Childhood. Vol. I. Evaluation. Charles C. Thomas, Publisher, Springfield, Illinois.

SHAPIRO, A., H. KOSTER, D. RITTENBERG AND R. SCHOENHEIMER 1936 The origin of fecal fat in the absence of bile, studied with deuterium as an indicator. Am. J. Physiol., vol. 117, p. 525.

SPERRY, W. M., AND W. R. BLOOR 1924 Fat excretion. II. The quantitative relations of the fecal lipoids. J. Biol. Chem., vol. 60, p. 261.

TEAGUE, D. MAXWELL, HARRY GALBRAITH, FRANCES COPE HUMMEL, HAROLD H. WILLIAMS AND ICIE G. MACY 1942 Effects of desiccation procedures on the chemical composition of feces, urine and milk. J. Lab. Clin. Med., vol. 28, p. 343.

TELFER, S. V. 1930 Mineral metabolism in infancy. Part I. The mineral constituents of human milk and of cow's milk. Glasgow Med. J., vol. 113, p. 246.

TIDWELL, HERBERT C., AND L. EMMETT HOLT, JR. 1935 The estimation of the total lipids and the lipid partition in feces. J. Biol. Chem., vol. 112, p. 605.

TIDWELL, H. C., L. E. HOLT, JR., H. L. FARROW ANL SARAH NEALE 1935 Studies in fat metabolism. II. Fat absorption in premature infants and twins. J. Pediatrics, vol. 6, p. 481.

WANG, CHI CHE, CORINNE HOGDEN AND IDA GENTHER 1939 Metabolic study of five children with nephrotic syndrome. II. Fat and protein metabolism and creatinine and creatine output. Am. J. Dis. Child., vol. 58, p. 29.

THE EFFECT OF HIGH LEVELS OF PANTOTHENIC ACID ON REPRODUCTION IN THE RAT AND THE MOUSE

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It has been reported that the addition of calcium pantothenate to the diet of hens whose regular ration was well balanced and apparently adequate in every respect resulted in an increased hatchability in the eggs of these fowls as compared with eggs from hens not receiving the vitamin supplement. It was also shown in the same reports that the relative sizes of the brain and heart were affected in the embryos which developed from the pantothenic acid enriched eggs (Taylor et al., '41 a, b).

The oviparous as compared with the viviparous embryo is, of course, confronted with special conditions with regard to food supply during development. It was therefore of interest to determine if supplements of calcium pantothenate to the stock ration of mammalian forms would have comparable effects on the survival and growth of the embryo.

EXPERIMENTAL

Experiment 1

Albino rats of the Wistar strain which were reared in our laboratory were used in this experiment. Sixty breeding females all of which were about 5 months old were separated into a control group of twenty-five and an experimental group of thirty-five animals.

The controls were continued on the stock ration of commercial dog chow [1] which contained pantothenic acid to the extent of 14 μg. per gram of the ration. The experimental group was maintained on the commercial dog chow supplemented with 100 μg. of calcium pantothenate per gram of ration. After the rats had been on this diet for 2 weeks males were introduced to both groups.

The experiment was continued for a total period of 50 days, at the end of which time sixteen litters had been obtained from the control and twenty-two litters from the experimental group.

Day-old rats produced by the control and experimental animals were utilized for the brain and heart weight determinations. Litters, from six rats of each group, that were born between the twenty-eighth and forty-fourth days of the experiment were represented in this study. Dissections were carefully made with the aid of a binocular dissecting microscope, and the organs were weighed individually immediately after removal in special light-weight containers which were fitted with air-tight covers. A total of about a hundred offspring were investigated in this respect but the results reported here are based on about half that number since it was found necessary to select from the two groups day-old rats in pairs of equal weights.

Experiment 2

Mice of the dba strain were used in this study. Forty-two young breeding females, about 3 months old, were divided into two equal groups. The controls were maintained on the stock ration of a commercial dog chow. The experimental animals were fed the stock ration to which had been added 100 μg. of calcium pantothenate per gram of food. Males were introduced 1 week after the experiment had been initiated. The study was continued for a total of 50 days, at the end of which time twenty-four litters had been produced by the

[1] Purina.

controls and twenty-two litters by the animals on the vitamin enriched ration.

Experiment 3

This study utilized dba mice and was in all respects similar to the preceding experiment except that the experimental animals received the vitamin supplement by subdermal injection. Each mouse was injected daily with 400 μg. of calcium pantothenate dissolved in ¼ cc. of physiological salt solution. The controls were injected with the salt solution alone.

This experiment was continued for 33 days, at the end of which time each group had produced sixteen litters.

RESULTS AND DISCUSSION

Table 1 contains the results obtained on the effect on litter size of calcium pantothenate supplements in the diet. It will be noted that in all three of the experiments there was substantial agreement in this respect.

TABLE 1

The effect of calcium pantothenate supplements on litter size.

	NO. OF LITTERS	OFFSPRING		STANDARD DEVIATION	P.E. DIFF. BETWEEN MEANS	$\frac{D}{PE}$	RELATIVE LITTER SIZE C = 100
		Total	Average per litter				
Reproducing rats — 100 μg. per gram of ration							
Control	16	95	5.9	.80	.240	6.5	...
Experimental	22	165	7.5	1.40			127
Reproducing mice — 100 μg. per gram of ration							
Control	24	125	5.2	.99	.084	15.5	...
Experimental	22	142	6.50	1.00			124
Reproducing mice — 400 μg. per day by subdermal injection							
Control	18	93	5.2	1.03	.224	4.9	...
Experimental	16	100	6.3	1.27			121

There was an increase of 21 to 27% in litter size in the experimental groups as compared to that in the controls. This was true whether the animals were rats or mice, and whether in the instance of the mice the additional pantothenic acid was received orally or by subdermal injection.

These data gain additional validity in view of the fact that similar results have been obtained with chick embryos (Taylor et al., '41 a, b).

As to the mechanism back of this effect, nothing is known at present. With regard to the mammal, the extra pantothenic acid could serve either to stimulate more ovulations or to enable more of the fertilized ova to survive to birth. It is well-known that in such species as the rat many more ova may be fertilized than survive to parturition. There is a sort of natural selection operating during early development. It is common to find embryos in the process of resorption when the uterus of a pregnant rat or mouse is examined.

In view of the results reported for hens' eggs, it seems likely that the pantothenic acid supplement affects the survival of the discharged ova rather than changes in the number of eggs produced by the ovaries.

TABLE 2

The effect of a pantothenic acid supplemented diet on brain and heart size in the offspring of the rat.

	NO. OF DAY-OLD RATS	AVERAGE BODY WT.	AVERAGE ORGAN WT.	ORGAN IN % OF BODY WEIGHT	STANDARD DEVIATION	P. E. DIFF. BETWEEN MEANS	$\frac{D}{PE}$	RELATIVE ORGAN SIZE WHERE CONTROL = 100
		gm.	*gm.*					
Brain								
Control	24	5.67	.218	3.84	.592			...
Experimental	24	5.67	.182	3.21	.387	.166	3.8	83.6
Heart								
Control	24	5.67	.0805	1.42	.249			...
Experimental	24	5.67	.0675	1.19	.260	.040	5.7	83.8

Table 2 records the brain and heart weights for day-old rats produced by the experimental and control animals. There was a definite tendency for the brains and hearts of the rats produced by the females on the pantothenic acid enriched diet to be relatively small in comparison with these organs in the offspring produced by the controls. While the number of

animals used in these determinations was not sufficiently large to permit generalization, the data are statistically valid as far as they go and agree in general with comparable data obtained for the chick experiments.

Some results of a preliminary nature have been reported for nicotinic acid, riboflavin and thiamine with regard to their effect on the development of the chick embryo. The use of large doses of these B vitamins also appears to affect the relative sizes of some of the chick organs (Taylor et al., '41 b).

SUMMARY

Groups of breeding albino rats and mice of the dba strain were maintained on a commercial dog chow with and without a 100 μg. per gram supplement of calcium pantothenate.

During the course of the experiment fifty-eight control litters averaged 5.4 offspring per litter, and sixty litters produced by the animals on the vitamin enriched diet averaged 6.7 offspring per litter — an average increase of 24% for the latter.

A study of brain and heart weights in day-old rats produced by control and experimental groups indicated that the calcium pantothenate supplemented diet was associated with a relatively smaller brain and heart in the offspring.

LITERATURE CITED

TAYLOR, ALFRED, J. THACKER AND D. PENNINGTON 1941 a The effect of increased pantothenic acid in the egg on the development of the chick embryo. Science, vol. 94, pp. 542–543.

TAYLOR, ALFRED, H. K. MITCHELL AND M. A. POLLACK 1941 b Modification of "B vitamin" content during embryological development. Univ. of Texas Pub. No. 4137, pp. 67–80.

THIAMINE ASSAYS OF FOODS USING THE RAT-GROWTH METHOD [1]

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Although chemical methods for the determination of thiamine are being improved, a rat-growth method that permits statistical analyses of the results continues to be of great value for vitamin assays of foods, especially foods of low thiamine content. The Committee on Vitamin Assay Methods for the State Agricultural Experiment Stations and the United States Department of Agriculture has recently recommended that biological assays on different types of foods should be used to check the chemical methods.

The diet for the method previously described (Miller, '39) contains autoclaved yeast as a source of the B vitamins other than thiamine. Since that time, it has been clearly established that pantothenic acid, pyridoxine, and choline are needed by the growing rat and it seemed advisable to retest the growth response of groups of rats with the addition of these three substances to the diet previously used.

This paper presents (1) the results of feeding groups of rats a thiamine-free diet supplemented with the pure vitamin (obtained from two chemical companies, each product having been synthesized by a different process), with and without supplements of pantothenic acid, pyridoxine, and choline; (2) the results of using the same rats for a second test after redepletion of thiamine stores; and (3) a comparison of the thiamine

[1] Published with the permission of the director of the Hawaii Agricultural Experiment Station as technical paper no. 105.

values of a food determined after both a first and a second depletion of body stores of thiamine, with and without supplements of pantothenic acid, pyridoxine, and choline.

PROCEDURE

The Chase and Sherman basal diet previously used was modified by substitution of cottonseed oil (part in hydrogenated form) for butter, and an increase in the amount of cod liver oil to lessen the cost. The use of sugar instead of cornstarch recommended by the U.S.P. Advisory Board ('39) proved unsatisfactory in this climate of relatively high humidity because the diet becomes too sticky. As no difficulties with refection have been experienced in recent years in our laboratory, cornstarch was used as previously.

At 21 days of age, sixty rats were weaned and placed on a diet having the following percentage composition: vitamin B-free casein, 18; salt mixture,[2] 2.5; cod liver oil, 3; cottonseed oil,[3] 2; hydrogenated cottonseed oil,[4] 4; cornstarch, 55.5; and autoclaved yeast,[5] 15. The rats were housed and weighed as previously described. All of the rats were placed on the experimental diet within a period of 21 days. The mean weight of the sixty rats used for the experiment was 43.4 ± 0.46 gm.[6] When depleted of their vitamin stores at an average of 18.8 days, their mean weight was 74.6 ± 0.73 gm. All rats were started on the supplements within a period of 24 days.

One animal from each litter was used as a negative control and the remainder were divided among three groups of sixteen rats each, there being eight females and eight males in each group. The groups were fed the supplements indicated in table 1.

[2] Salt mixture 351, Hubbell, Mendel and Wakeman ('37).
[3] Wesson.
[4] Crisco.
[5] Fleischmann's yeast, type 188 autoclaved at 15 pounds pressure for 5 hours. As this type of yeast has not been available since May, 1942, type 2019 which has a higher thiamine content has been used successfully.
[6] Standard error of the mean.

TABLE 1

Summary of the results of feeding groups of rats thiamine with and without supplements of pantothenic acid, pyridoxine, and choline both after a first and a second depletion of thiamine stores.

DAILY SUPPLEMENTS	NUMBER OF ANIMALS	AVERAGE WEIGHTS			MEAN GAINS IN 3 WEEKS		
		At start	At depletion	Final weights		Standard deviation	"t" value
		gm.	*gm.*	*gm.*	*gm.*		
Series I After first depletion of thiamine stores							
Negative controls	12	44	77	63	−14		
a. Thiamine,[1] 3 μg.	16	43	72	106	34 ± 1.7	6.9	20.0
b. Thiamine,[2] 3 μg.	16	43	75	111	36 ± 2.6	10.5	13.8
c. Thiamine,[2] 3 μg. 15 μg. pyridoxine, and 50 μg. pantothenic acid [3]	16	43	75	116	41 ± 2.8	11.0	14.6
Series II After second depletion of thiamine stores							
a. Thiamine,[1] 4 μg.	16	108	108	148	40 ± 2.2	8.9	18.2
b. Thiamine,[2] 4 μg.	16	112	110	149	39 ± 2.2	8.9	17.7
c. Thiamine,[2] 4 μg. 15 μg. pyridoxine, and 50 μg. pantothenic acid [3]	15	114	112	152	40 ± 2.1	8.1	19.0

[1] Winthrop.
[2] Merck.
[3] One gram of choline per 1000 gm. of diet was added to this diet.

One of the thiamine preparations [7] was given in a 10% alcoholic solution containing 6 μg. in 0.1 ml. A fresh solution of the vitamin was made each week, placed in a brown glass bottle and kept in the refrigerator, only the small portion needed for each day's feedings being removed at one time. The other thiamine solution [8] was prepared exactly as directed by the U.S.P. Vitamin Advisory Board ('39). The thiamine supplements were fed by pipette three times a week in amounts to provide 3 μg. per day (except Sunday).

On the basis of published data it was believed that 15% of autoclaved yeast in the diet supplied ample quantities of the other B vitamins but that possibly it contained insufficient

[7] Winthrop.
[8] U.S.P. standard Merck.

quantities of pantothenic acid, pyridoxine, and choline. The autoclaved yeast used contained about 40 μg. of pantothenic acid per gram.[9] Judging from a number of published reports available at the time the experiments were initiated, it was estimated that these substances should be added in the following quantities: 50 μg. pantothenic acid and 15 μg. pyridoxine per day (except Sunday), and 1 gm. of choline per 1000 gm. of basal diet. Calcium pantothenate and pyridoxine hydrochloride were prepared in faintly acidified 10% alcoholic solutions and were fed by pipette three times a week.

The average of the weights of the rats for the last 3 days of the experiment was used as the final weight.

After 3 weeks of supplementary feeding, the first experiment was completed and no supplements were given. The rats were weighed every day; when the weights were stationary or there were slight losses for 3 to 4 consecutive days, the rats were considered redepleted. It took an average of 5 days for redepletion of all the animals. One unusually large rat fed the extra supplements was discarded. All rats were continued in the same groups as for the first experiment and supplements were again fed for a period of 3 weeks. Because it was assumed that the larger rats of series II would need more thiamine, all rats were fed 4 μg. thiamine daily (except Sunday) and one group was fed the diet containing choline plus the same quantities of pyridoxine and pantothenic acid, as in series I. The weights of the animals at the end of the second depletion were less uniform (S.E. $\pm$ 1.6) than those after the first.

RESULTS AND DISCUSSION

The mean gains of the three groups of rats are given in table 1 with other pertinent data. The standard errors and the "t" values indicate a high degree of reliability.

The difference of 2 gm. between the mean gains for group a, series I, and those for group b is obviously not significant.

[9] The author is indebted to Dr. T. H. Jukes of the University of California, Davis, for determining the pantothenic acid content of the autoclaved yeast.

Statistical analysis of the difference between the mean gains of group b, series I, fed the thiamine as the only supplement, and those for group c fed supplements of thiamine, pantothenic acid, pyridoxine, and choline, shows that the difference is not significant at the 5% level.

After a second depletion of thiamine stores and for a second period of supplementary feeding (table 1, series II) the mean gains of the three groups were practically the same.

To determine the quantity of pantothenic acid to be fed daily, it was calculated that if the rats ate 5 gm. of diet each day they would receive 30 μg. of pantothenic acid daily from the basal diet and that 50 μg. daily in addition should furnish the 80 μg. recommended by Unna ('40) as being the quantity necessary per rat per day for optimal growth. Actually only one rat ate as low an average as 5 gm. of basal diet per day and the average for both males and females was more than 6 gm. of basal diet daily for the 3-week test period.

Since the work here reported was completed, Unna and Richards ('42) have found that weanling rats (3 weeks of age) require 80 to 100 μg. of pantothenic acid for optimum growth and that this requirement decreases to 25 μg. at 10 weeks of age. Our rats were approximately 5½ weeks of age when started on the supplements after the first depletion, and about 9 weeks when started on the supplements after the second depletion.

In the light of Unna and Richards' report, the difference between the mean gains of the rats with and without supplements in series I might be interpreted as the result of insufficient pantothenic acid at that stage of growth, especially as there was no difference in series II when the need of the rats for pantothenic acid was less. However, since the rats were receiving daily more than 80 μg. of pantothenic acid from the diet plus the supplement at the age of 5 to 8 weeks when their requirement should be less than that amount, the conclusion that there is no significant difference between the

weight gains of the rats with and without the supplements is believed to be correct.

From the data presented in table 1, it is concluded that the method and diet previously described are satisfactory for the determination of the thiamine content of foods, and that the rats used for one test may be redepleted of their thiamine stores and used for a second test.

TABLE 2

Summary of the results of feeding rice polish to rats as the sole source of thiamine.

DAILY SUPPLEMENTS	NUMBER OF ANIMALS	AVERAGE WEIGHTS			MEAN GAINS IN 3 WEEKS		Thiamine per 100 gm.
		At start	At depletion	Final weights		Standard deviation	
		gm.	*gm.*	*gm.*	*gm.*		*μg.*
Series I							
Group A — after first depletion of thiamine stores							
Negative controls	9	42	66	45	−21		
Thiamine, 3 μg.	10	41	67	87	20 ± 2.6	8.1	
Rice polish, 0.1 gm.	13	43	71	96	25 ± 1.8	6.4	3600
Group B — after second depletion of thiamine stores							
Negative controls	5	105	99	71	−28		
Thiamine, 3 μg.	11	95	90	105	15 ± 2.4	8.0	
Rice polish, 0.1 gm.	13	97	92	114	22 ± 2.6	9.5	3630
Series II (with supplements of pantothenic acid, pyridoxine, and choline)							
Group A — after first depletion of thiamine stores							
Negative controls	17	42	72	50	−22		
Thiamine, 3 μg.	14	42	73	98	25 ± 2.9	11.0	
Rice polish, 0.1 gm.	12	40	68	98	30 ± 2.8	9.6	3600
Group B — after second depletion of thiamine stores							
Thiamine, 4 μg.	13	102	98	125	27 ± 2.3	8.4	
Rice polish, 0.1 gm.	12	103	98	118	20 ± 2.5	8.6	3332

DETERMINATION OF THE THIAMINE CONTENT OF A FOOD BY THE METHOD RECOMMENDED

To test further the conclusion that supplements to the thiamine-free diet of pantothenic acid, pyridoxine, and choline are unnecessary and that rats depleted of their thiamine stores for a second time may be used for determining the thiamine content of food, two series of experiments were carried out, using rice polish.[10]

The basal diet without supplements was used for series I, and with supplements (pyridoxine, pantothenic acid, and choline), as described in this paper, for series II. In each series, groups of rats were used after both a first and a second depletion of thiamine stores. For both series the rats were part of a larger group used to test other foods as well. A curve of response to graded doses of thiamine after a second depletion of thiamine stores was established as for the first depletion (Miller, '39), and this was used for calculating the thiamine values of the rice polish.

The results of these experiments, using 129 rats, are summarized in table 2. The calculated thiamine values of the rice polish vary but little from 3332 μg. to 3630 μg. per 100 gm. The difference between any two of the values is less than 10%, which is probably within the error of the biological method.

SUMMARY AND CONCLUSIONS

1. The growth response of rats fed a thiamine-free diet in which autoclaved yeast provided other members of the vitamin B complex was not significantly improved by supplements of pantothenic acid, pyridoxine, and choline in addition to thiamine.

2. Rats used for a 3-week test period may be redepleted of thiamine stores and used for a second test, thus saving time and money.

3. The thiamine values of a food (rice polish) determined by the growth response of rats after both a first and a second

[10] The rice polish — a fine cream-colored, flour-like product — was obtained from a local rice mill where it was called "rice middlings."

depletion of thiamine stores were the same, whether or not the diet was supplemented with pantothenic acid, pyridoxine, and choline.

LITERATURE CITED

COMMITTEE OF REVISION OF THE U. S. PHARMACOPOEIA 1939 Reference standard and assay for thiamin chloride or vitamin B_1. J. Am. Pharm. Assoc., vol. 28, p. 267.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

MILLER, C. D. 1939 Determination of a curve of response to synthetic crystalline thiamin, for use in the vitamin B_1 assay of foods by the rat-growth method. J. Nutrition, vol. 17, p. 535.

UNNA, K. 1940 Pantothenic acid requirement of the rat. J. Nutrition, vol. 20, p. 565.

UNNA, K., AND G. V. RICHARDS 1942 Relationship between pantothenic acid requirement and age in the rat. J. Nutrition, vol. 23, p. 545.

THE EFFECT OF PANTOTHENIC ACID ON THE RATE OF INTESTINAL ABSORPTION OF GALACTOSE IN THE RAT [1]

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Russell and Nasset ('41) reported that supplements of fresh yeast, dried yeast, or yeast extract when added to a normal dog's diet increased the rate of digestion and absorption of its carbohydrates. Similar effects were produced by the addition of calcium pantothenate. Severe gastrointestinal disturbances have been noted in animals with pantothenic acid deficiency (Morgan and Simms, '40; Schaefer, McKibbin and Elvehjem, '42). Numerous observations have been made of the occurrence of hemorrhage, atrophy and necrosis of the adrenal cortex in pantothenic acid deficiency (Daft, Sebrell, Babcock and Jukes, '40). Verzar ('36) has suggested that the adrenal cortex has a specific role in the intestinal absorption of hexose sugars. This provides an indirect suggestion that in pantothenic acid deficiency an impairment of intestinal absorption might arise from adrenal cortical insufficiency. A recent report (Free and Leonards, '42) from this laboratory indicated that in vitamin B complex deficiency in rats, a decrease in intestinal absorption occurred. The present report describes studies on the effects of both pantothenic acid deficiency and an excess of calcium pantothenate on intestinal absorption.

[1] This investigation was supported by a grant from the Williams-Waterman Fund of the Research Corporation.

EXPERIMENTAL

Pantothenic acid deficiency

Albino and piebald rats reared in the department colony were placed on a diet having a percentage composition of alcohol-extracted casein, 18; sucrose, 45; starch, 23; cottonseed oil, 8; Mendel-Hubbell-Wakeman salt mixture ('37), 4; and cod liver oil, 2; to 100 parts of this mixture was added 0.2 part of choline chloride. On alternate days the animals received a supplement of crystalline vitamins which provided a daily intake of thiamine 40 μg., riboflavin 25 μg., and pyridoxine 20 μg.

Each experimental animal was "paired" with a litter mate of the same sex which received the same diet and supplement with the daily addition of 100 μg. of calcium pantothenate.[2] The food intake of the control member of the pair was regulated so that its weight was kept nearly the same as that of the experimental member. Typical symptoms of pantothenic acid deficiency developed, including a graying of the black fur of the piebald rats and an accumulation of red material around the nose and on the whiskers, particularly evident in the white rats. This pigment has been identified as a porphyrin excreted by the Harderian glands (McElroy, Salomon, Figge and Cowgill, '41). None of the control animals which received calcium pantothenate supplements showed graying or pigment accumulation. Intestinal absorption measurements were made after the animals had been on the experimental diet for a period of from 7 to 9 weeks.

Pantothenic acid excess

In these experiments young albino rats were employed. These animals were of the same strain as the albinos used in the preceding experiment and were reared on a stock diet of dog chow. They were then divided into two groups. One-half of the animals in the first group received a daily supple-

[2] We are indebted to Merck and Company, Rahway, N. J., for generous supplies of the vitamins employed in this study.

ment of 300 μg. of calcium pantothenate; the other half served as controls. The other group was similarly treated except that they received 1000 μg. of the vitamin daily. The animals received these supplements for 6 days during which time they were kept on the stock diet. Food was removed 24 hours prior to the absorption measurements and 12 hours later the animals were given their final supplement of calcium pantothenate which in this instance was twice that of the preceding days. The control animals were not supplemented.

The technics for the measurements of intestinal absorption and blood galactose level employed in this study were identical with those already described (Free and Leonards, '42). Galactose was employed as the test substance. Exactly 1 hour after ingesting a fixed amount of the sugar the animals were sacrificed and the amounts of galactose remaining in the stomach and intestines were determined, as well as the concentration of galactose in the blood. The coefficient of absorption is a value expressing the milligrams of galactose absorbed per 100 gm. of body weight per hour. The level of galactose in the blood is used as an indirect measure of the rate of intestinal absorption of the sugar.

RESULTS

The individual findings of the absorption measurements of each of the pantothenic acid deficient rats, as well as those of their "paired" controls are given in table 1. The initial and final body weights are given in the table but they do not indicate the fact that the rats gained most of their weight during the first 4 weeks after which their weights remained essentially unchanged. Since the rate of gastric emptying of the sugar is an important factor in this type of measurement of intestinal absorption, it is important to note that the gastric residue is nearly the same in both groups of animals, being 36% of the ingested dose in the deficient rats and 33% in the controls. The variations of both absorption coefficients and blood galactose levels among the members of each group are considerable, but on the average the coefficient of absorp-

TABLE 1

The effect of pantothenic acid deficiency on absorption coefficients and blood galactose levels.

RAT			DEFICIENT ANIMALS						CONTROL ANIMALS						DIFFERENCE (CONTROL MINUS DEFICIENT)	
			Body weight		Galactose				Body weight		Galactose					
Pair	Sex	Strain	Initial	Final	In stomach [1]	In intestines [1]	Absorption coefficient	In blood	Initial	Final	In stomach [1]	In intestines [1]	Absorption coefficient	In blood	Absorption coefficient	Blood galactose
			gm.	*gm.*	%	%	*mg./100 gm./hr.*	*mg./100 cc.*	*gm.*	*gm.*	%	%	*mg./100 gm./hr.*	*mg./100 cc.*		
1	F	W	42	97	49	17	214	452	43	83	36	8	336	...	+122	...
2	F	W	50	81	26	17	342	616	47	82	28	8	384	550	+ 42	— 66
3	F	W	47	90	40	11	294	468	48	86	46	16	228	451	— 66	— 17
4	F	W	44	88	38	11	306	...	44	82	42	4	312	...	+ 6	...
5	F	W	40	76	29	14	342	625	46	76	35	11	324	648	— 18	+ 23
6	M	W	47	113	54	13	198	465	43	103	45	8	282	474	+ 84	+ 9
7	F	W	40	71	43	9	288	447	38	61	26	6	408	518	+120	+ 71
8	M	W	37	62	23	9	408	572	31	56	3	9	528	657	+120	+ 85
9	M	W	41	109	49	14	222	418	37	97	45	7	288	410	+ 66	— 8
10	F	P	35	68	22	11	402	753	36	61	19	9	432	668	+ 30	— 85
11	F	P	32	74	39	16	270	445	31	71	22	11	402	437	+132	— 8
12	M	P	32	69	42	17	246	650	33	68	27	10	378	673	+132	+ 23
13	M	P	42	95	33	13	324	575	41	91	32	10	348	540	+ 24	— 35
14	F	P	42	77	51	21	168	392	42	74	42	11	282	388	+114	— 4
15	M	P	33	50	7	15	468	708	35	52	27	12	366	580	—102	—128
16	F	P	35	75	31	19	300	596	34	70	30	11	354	477	+ 54	—119
17	M	P	38	64	41	16	258	517	33	64	56	9	210	432	— 48	— 85
18	M	P	38	61	34	10	336	520	34	65	37	8	330	575	— 6	+ 55
Average			40	79	36	14	299	542	39	75	33	9	344	530		

[1] Fraction of ingested sugar found in the organ.

tion of galactose is 299 mg./100 gm./hr. for the deficient animals and 344 mg./100 gm./hr. for the controls. The blood galactose levels of the deficient animals averaged 542 mg./100 cc., whereas the control rats averaged 530 mg. of galactose per 100 cc. of blood.

The results of the study in which the animals were given excesses of calcium pantothenate are summarized in table 2.

TABLE 2

The effect of calcium pantothenate excess on the absorption coefficients and blood galactose levels.

SUPPLEMENT	NUMBER OF RATS	BODY WEIGHT	GALACTOSE			
			In stomach [1]	In intestines [1]	Absorption coefficient	In blood
		gm.	%	%	*mg./100 gm./hr.*	*mg./100 cc.*
Controls	9	143	32	18	301	513
300 μg. calcium pantothenate	9	147	31	18	305	514
Controls	10	123	34	19	282	423
1000 μg. calcium pantothenate	10	131	31	21	288	433

[1] Fraction of ingested sugar found in the organ.

The variations in coefficients of absorption and blood galactose levels were somewhat less in this group of animals than in the preceding series. The average absorption coefficients and blood galactose levels of the animals receiving the daily supplement of 300 μg. of calcium pantothenate, as well as those receiving 1000 μg., corresponded very closely to the average values of their respective control groups.

In Russell and Nasset's ('41) experiments the effect of calcium pantothenate was exerted in the presence of additional quantities of other B complex vitamins. To simulate these conditions in the rat studies it was considered desirable to

conduct an experiment in which additional amounts of thiamine, riboflavin and pyridoxine were administered both with and without calcium pantothenate. Fifteen male albino rats of similar weights were divided into three equal groups. The first group received no supplements and served as controls, the second group received daily supplements of thiamine 250 μg., riboflavin 250 μg. and pyridoxine 200 μg. over a period of 6 days; and the third group received the same supplements given to the second group along with 1000 μg. of calcium pantothenate daily. The animals were kept on the stock diet during this period. The absorption coefficients and blood galactose levels of the three groups showed no significant differences. In fact the animals that were not supplemented had slightly higher absorption coefficients.

DISCUSSION

A comparison of the absorption coefficients of the animals deficient in pantothenic acid with their litter mate pair-fed controls, shows no striking difference, although the average absorption coefficient for the deficient animals is less than that of the controls by 45 mg. per 100 gm. per hour. This difference amounts to 15%. During the first weeks of pantothenic acid deficiency in the rat, the animal gains weight and then gradually reaches a constant level. At a somewhat variable period after the weight has been stabilized, a crisis occurs at which time adrenal cortical hemorrhage and necrosis are observed, and shortly thereafter death ensues. The absorption studies in our animals were made at a time when the weight was stabilized, prior to the occurrence of any crisis. This was attested to by the general behavior of the animals at the time of the studies and also by the absence of macroscopic evidence of adrenal hemorrhage. The extent of the decrease in intestinal absorption in these studies does not represent evidence of gross intestinal dysfunction. However, the findings do not lend themselves to a prediction of intestinal function in respect to absorption in acute stages of pantothenic acid deficiency. The blood galactose levels observed in the deficient

rats are somewhat higher than would be predicted from the absorption coefficients. This might indicate that the rate of removal of absorbed galactose from the blood is somewhat slower than normal. This may be due to an impairment in either the metabolism or excretion of the sugar.

The data obtained from the rats on the normal diet which received excesses of 300 μg. and 1000 μg. of calcium pantothenate per day, indicate that the additional pantothenic acid had no influence on the rate of intestinal absorption of galactose. These observations do not support the findings of Russell and Nasset ('41) in which it was noted that calcium pantothenate caused a marked increase in the rate of carbohydrate digestion and absorption in the dog. It should be noted that our experiments were carried out under a set of conditions in which digestion played no part, whereas the experiments of Russell and Nasset were of such a nature that the measurements of the rate of intestinal absorption were dependent on the rate of digestion. Whether this factor of digestion is responsible for the different results remains to be ascertained.

SUMMARY

The rate of intestinal absorption was measured in eighteen litter mate pairs of rats in which one member of the pair received a synthetic diet deficient in pantothenic acid and the other member received the same diet supplemented with 100 μg of calcium pantothenate daily. The rate of intestinal absorption of the pair-fed litter mate controls averaged 15% more than that of the deficient animals. One hour after the ingestion of the sugar blood galactose levels were essentially the same in both groups of rats.

Rats maintained on an adequate stock diet were supplemented with 300 μg. and 1000 μg. of calcium pantothenate daily. This supplementation of a normal diet with additional calcium pantothenate had no effect on the rate of intestinal absorption of galactose.

LITERATURE CITED

DAFT, F. S., W. H. SEBRELL, S. H. BABCOCK, JR., AND T. H. JUKES 1940 Effect of synthetic pantothenic acid on adrenal hemorrhage, atrophy, and necrosis in rats. Pub. Health Reports, vol. 55, p. 1333.

FREE, A. H., AND J. R. LEONARDS 1942 The effect of vitamin B deficiency on the intestinal absorption of galactose in the rat. J. Nutrition, vol. 24, p. 495.

MCELROY, L. W., K. SALOMON, F. H. J. FIGGE AND G. R. COWGILL 1941 On the porphyrin nature of the fluorescent "blood caked" whiskers of pantothenic acid deficient rats. Science, vol. 94, p. 467.

MENDEL, L. B., R. B. HUBBELL AND A. J. WAKEMAN 1937 The influence of some commonly used salt mixtures upon growth and bone development of the albino rat. J. Nutrition, vol. 14, p. 261.

MORGAN, A. F., AND H. D. SIMMS 1940 Greying of fur and other disturbances in several species due to a vitamin deficiency. J. Nutrition, vol. 19, p. 233.

RUSSELL, R. A., AND E. S. NASSET 1941 The effects of various vitamin supplements and of whole yeast on the digestion and absorption of the carbohydrate of a complete diet. J. Nutrition, vol. 22, p. 287.

SCHAEFER, A. E., J. M. MCKIBBIN AND C. A. ELVEHJEM 1942 Pantothenic acid deficiency studies in dogs. J. Biol. Chem., vol. 143, p. 321.

VERZAR, F. 1936 Absorption from the intestine. Longmans, Green and Co., New York.